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Soluble Recombinant Botulinum Toxin Proteins  
~~MULTIVALENT VACCINE FOR CLOSTRIDIUM BOTULINUM NEUROTOXIN~~ 08 704159

This application is a Continuation-In-Part of copending Application Serial No. 08/405,496, filed March 16, 1995.

## FIELD OF THE INVENTION

5 The present invention relates to the isolation of polypeptides derived from *Clostridium botulinum* neurotoxins and the use thereof as immunogens for the production of vaccines, including multivalent vaccines, and antitoxins.

## BACKGROUND OF THE INVENTION

10 The genus *Clostridium* is comprised of gram-positive, anaerobic, spore-forming bacilli. The natural habitat of these organisms is the environment and the intestinal tracts of humans and other animals. Indeed, clostridia are ubiquitous; they are commonly found in soil, dust, sewage, marine sediments, decaying vegetation, and mud. [See e.g., P.H.A. Sneath *et al.*, "*Clostridium*," *Bergey's Manual® of Systematic Bacteriology*, Vol. 2, pp. 1141-1200, Williams & Wilkins (1986).] Despite the  
15 identification of approximately 100 species of *Clostridium*, only a small number have been recognized as etiologic agents of medical and veterinary importance. Nonetheless, these species are associated with very serious diseases, including botulism, tetanus, anaerobic cellulitis, gas gangrene, bacteremia, pseudomembranous colitis, and clostridial gastroenteritis. Table 1 lists some of the species of medical and  
20 veterinary importance and the diseases with which they are associated. As virtually all of these species have been isolated from fecal samples of apparently healthy persons, some of these isolates may be transient, rather than permanent residents of the colonic flora.

TABLE 1

*Clostridium* Species Of Medical And Veterinary Importance\*

Species	Disease
<i>C. aminovalericum</i>	Bacteriuria (pregnant women)
<i>C. argentinense</i>	Infected wounds; Bacteremia; Botulism; Infections of amniotic fluid
<i>C. baratii</i>	Infected war wounds; Peritonitis; Infectious processes of the eye, ear and prostate
<i>C. beijerinckii</i>	Infected wounds
<i>C. bifermentans</i>	Infected wounds; Abscesses; Gas Gangrene; Bacteremia
<i>C. botulinum</i>	Food poisoning; Botulism (wound, food, infant)
<i>C. butyricum</i>	Urinary tract, lower respiratory tract, pleural cavity, and abdominal infections; Infected wounds; Abscesses; Bacteremia
<i>C. cadaveris</i>	Abscesses; Infected wounds
<i>C. carnis</i>	Soft tissue infections; Bacteremia
<i>C. chauvoei</i>	Blackleg
<i>C. clostridioforme</i>	Abdominal, cervical, scrotal, pleural, and other infections; Septicemia; Peritonitis; Appendicitis
<i>C. cochlearium</i>	Isolated from human disease processes, but role in disease unknown.
<i>C. difficile</i>	Antimicrobial-associated diarrhea; Pseudomembranous enterocolitis; Bacteremia; Pyogenic infections
<i>C. fallax</i>	Soft tissue infections
<i>C. ghnoii</i>	Soft tissue infections
<i>C. glycolicum</i>	Wound infections; Abscesses; Peritonitis
<i>C. hastiforme</i>	Infected war wounds; Bacteremia; Abscesses
<i>C. histolyticum</i>	Infected war wounds; Gas gangrene; Gingival plaque isolate
<i>C. indolis</i>	Gastrointestinal tract infections
<i>C. innocuum</i>	Gastrointestinal tract infections; Empyema
<i>C. irregulare</i>	Penile lesions
<i>C. leptum</i>	Isolated from human disease processes, but role in disease unknown.
<i>C. limosum</i>	Bacteremia; Peritonitis; Pulmonary infections

TABLE 1

*Clostridium* Species Of Medical And Veterinary Importance\*

Species	Disease
<i>C. malenominatum</i>	Various infectious processes
<i>C. novyi</i>	Infected wounds; Gas gangrene; Blackleg, Big head (ovine); Redwater disease (bovine)
<i>C. oroticum</i>	Urinary tract infections; Rectal abscesses
<i>C. paraputrificum</i>	Bacteremia; Peritonitis; Infected wounds; Appendicitis
<i>C. perfringens</i>	Gas gangrene; Anaerobic cellulitis; Intra-abdominal abscesses; Soft tissue infections; Food poisoning; Necrotizing pneumonia; Empyema; Meningitis; Bacteremia; Uterine Infections; Enteritis necrotans; Lamb dysentery; Struck; Ovine Enterotoxemia;
<i>C. putrefaciens</i>	Bacteriuria (Pregnant women with bacteremia)
<i>C. putrificum</i>	Abscesses; Infected wounds; Bacteremia
<i>C. ramosum</i>	Infections of the abdominal cavity, genital tract, lung, and biliary tract; Bacteremia
<i>C. sartagoforme</i>	Isolated from human disease processes, but role in disease unknown.
<i>C. septicum</i>	Gas gangrene; Bacteremia; Suppurative infections; Necrotizing enterocolitis; Braxy
<i>C. sordellii</i>	Gas gangrene; Wound infections; Penile lesions; Bacteremia; Abscesses; Abdominal and vaginal infections
<i>C. sphenoides</i>	Appendicitis; Bacteremia; Bone and soft tissue infections; Intraperitoneal infections; Infected war wounds; Visceral gas gangrene; Renal abscesses
<i>C. sporogenes</i>	Gas gangrene; Bacteremia; Endocarditis; central nervous system and pleuropulmonary infections; Penile lesions; Infected war wounds; Other pyogenic infections
<i>C. subterminale</i>	Bacteremia; Empyema; Biliary tract, soft tissue and bone infections
<i>C. symbiosum</i>	Liver abscesses; Bacteremia; Infections resulting due to bowel flora
<i>C. tertium</i>	Gas gangrene; Appendicitis; Brain abscesses; Intestinal tract and soft tissue infections; Infected war wounds; Periodontitis; Bacteremia



TABLE 1

*Clostridium* Species Of Medical And Veterinary Importance\*

Species	Disease
<i>C. tetani</i>	Tetanus; Infected gums and teeth; Corneal ulcerations; Mastoid and middle ear infections; Intraperitoneal infections; Tetanus neonatorum; Postpartum uterine infections; Soft tissue infections, especially related to trauma (including abrasions and lacerations); Infections related to use of contaminated needles
<i>C. thermosaccharolyticum</i>	Isolated from human disease processes, but role in disease unknown.

\* Compiled from P.G. Engelkirk *et al.* "Classification", *Principles and Practice of Clinical Anaerobic Bacteriology*, pp. 22-23, Star Publishing Co., Belmont, CA (1992); J. Stephen and R.A. Petrowski, "Toxins Which Traverse Membranes and Deregulate Cells," in *Bacterial Toxins*, 2d ed., pp. 66-67, American Society for Microbiology (1986); R. Berkow and A.J. Fletcher (eds.), "Bacterial Diseases," *Merck Manual of Diagnosis and Therapy*, 16th ed., pp. 116-126, Merck Research Laboratories, Rahway, N.J. (1992); and O.H. Sigmund and C.M. Fraser (eds.), "Clostridial Infections," *Merck Veterinary Manual*, 5th ed., pp. 396-409, Merck & Co., Rahway, N.J. (1979).

In most cases, the pathogenicity of these organisms is related to the release of powerful exotoxins or highly destructive enzymes. Indeed, several species of the genus *Clostridium* produce toxins and other enzymes of great medical and veterinary significance. [C.L. Hatheway, *Clin. Microbiol. Rev.* 3:66-98 (1990).]

Perhaps because of their significance for human and veterinary medicine, much research has been conducted on these toxins, in particular those of *C. botulinum* and *C. difficile*.

### *C. botulinum*

Several strains of *Clostridium botulinum* produce toxins of significance to human and animal health. [C.L. Hatheway, *Clin. Microbiol. Rev.* 3:66-98 (1990)] The effects of these toxins range from diarrheal diseases that can cause destruction of the colon, to paralytic effects that can cause death. Particularly at risk for developing clostridial diseases are neonates and humans and animals in poor health (*e.g.*, those suffering from diseases associated with old age or immunodeficiency diseases).



*Clostridium botulinum* produces the most poisonous biological toxin known. The lethal human dose is a mere  $10^{-9}$  mg/kg bodyweight for toxin in the bloodstream. Botulinal toxin blocks nerve transmission to the muscles, resulting in flaccid paralysis. When the toxin reaches airway and respiratory muscles, it results in respiratory failure that can cause death. [S. Arnon, J. Infect. Dis. 154:201-206 (1986)]

*C. botulinum* spores are carried by dust and are found on vegetables taken from the soil, on fresh fruits, and on agricultural products such as honey. Under conditions favorable to the organism, the spores germinate to vegetative cells which produces toxin. [S. Arnon, Ann. Rev. Med. 31:541 (1980)]

Botulism disease may be grouped into four types, based on the method of introduction of toxin into the bloodstream. Food-borne botulism results from ingesting improperly preserved and inadequately heated food that contains botulinal toxin. There were 355 cases of food-borne botulism in the United States between 1976 and 1984. [K.L. MacDonald *et al.*, Am. J. Epidemiol. 124:794 (1986).] The death rate due to botulinal toxin is 12% and can be higher in particular risk groups. [C.O. Tacket *et al.*, Am. J. Med. 76:794 (1984).] Wound-induced botulism results from *C. botulinum* penetrating traumatized tissue and producing toxin that is absorbed into the bloodstream. Since 1950, thirty cases of wound botulism have been reported. [M.N. Swartz, "Anaerobic Spore-Forming Bacilli: The Clostridia," pp. 633-646, in B.D. Davis *et al.*, (eds.), *Microbiology*, 4th edition, J.B. Lippincott Co. (1990).] Inhalation botulism results when the toxin is inhaled. Inhalation botulism has been reported as the result of accidental exposure in the laboratory [E. Holzer, Med. Klin. 41:1735 (1962)] and could arise if the toxin is used as an agent of biological warfare [D.R. Franz *et al.*, in *Botulinum and Tetanus Neurotoxins*, B.R. DasGupta, ed., Plenum Press, New York (1993), pp. 473-476]. Infectious infant botulism results from *C. botulinum* colonization of the infant intestine with production of toxin and its absorption into the bloodstream. It is likely that the bacterium gains entry when

spores are ingested and subsequently germinate. [S. Arnon, J. Infect. Dis. 154:201 (1986).] There have been 500 cases reported since it was first recognized in 1976. [M.N. Swartz, *supra*.]

5 Infant botulism strikes infants who are three weeks to eleven months old (greater than 90% of the cases are infants less than six months). [S. Arnon, J. Infect. Dis. 154:201 (1986).] It is believed that infants are susceptible, due, in large part, to the absence of the full adult complement of intestinal microflora. The benign microflora present in the adult intestine provide an acidic environment that is not favorable to colonization by *C. botulinum*. Infants begin life with a sterile intestine 10 which is gradually colonized by microflora. Because of the limited microflora present in early infancy, the intestinal environment is not as acidic, allowing for *C. botulinum* spore germination, growth, and toxin production. In this regard, some adults who have undergone antibiotic therapy which alters intestinal microflora become more susceptible to botulism.

15 An additional factor accounting for infant susceptibility to infectious botulism is the immaturity of the infant immune system. The mature immune system is sensitized to bacterial antigens and produces protective antibodies. Secretory IgA produced in the adult intestine has the ability to agglutinate vegetative cells of *C. botulinum*. [S. Arnon, J. Infect. Dis. 154:201 (1986).] Secretory IgA may also act 20 by preventing intestinal bacteria and their products from crossing the cells of the intestine. [S. Arnon, Epidemiol. Rev. 3:45 (1981).] The infant immune system is not primed to do this.

25 Clinical symptoms of infant botulism range from mild paralysis, to moderate and severe paralysis requiring hospitalization, to fulminant paralysis, leading to sudden death. [S. Arnon, Epidemiol. Rev. 3:45 (1981).]

The chief therapy for severe infant botulism is ventilatory assistance using a mechanical respirator and concurrent elimination of toxin and bacteria using cathartics,

enemas, and gastric lavage. There were 68 hospitalizations in California for infant botulism in a single year with a total cost of over \$4 million for treatment.

[T.L. Frankovich and S. Arnon, West. J. Med. 154:103 (1991).]

5 Different strains of *Clostridium botulinum* each produce antigenically distinct toxin designated by the letters A-G. Serotype A toxin has been implicated in 26% of the cases of food botulism; types B, E and F have also been implicated in a smaller percentage of the food botulism cases [H. Sugiyama, Microbiol. Rev. 44:419 (1980)]. Wound botulism has been reportedly caused by only types A or B toxins [H. Sugiyama, *supra*].

10 Nearly all cases of infant botulism have been caused by bacteria producing either type A or type B toxin. (Exceptionally, one New Mexico case was caused by *Clostridium botulinum* producing type F toxin and another by *Clostridium botulinum* producing a type B-type F hybrid.) [S. Arnon, Epidemiol. Rev. 3:45 (1981).] Type C toxin affects waterfowl, cattle, horses and mink. Type D toxin affects cattle, and type E toxin affects both humans and birds.

15 A trivalent antitoxin derived from horse plasma is commercially available from Connaught Industries Ltd. as a therapy for toxin types A, B, and E. However, the antitoxin has several disadvantages. First, extremely large dosages must be injected intravenously and/or intramuscularly. Second, the antitoxin has serious side effects such as acute anaphylaxis which can lead to death, and serum sickness. Finally, the efficacy of the antitoxin is uncertain and the treatment is costly. [C.O. Tacket *et al.*, Am. J. Med. 76:794 (1984).]

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A heptavalent equine botulinal antitoxin which uses only the F(ab')<sub>2</sub> portion of the antibody molecule has been tested by the United States Military. [M. Balady, USAMRDC Newsletter, p. 6 (1991).] This was raised against impure toxoids in those large animals and is not a high titer preparation.

25

A pentavalent human antitoxin has been collected from immunized human subjects for use as a treatment for infant botulism. The supply of this antitoxin is

limited and cannot be expected to meet the needs of all individuals stricken with botulism disease. In addition, collection of human sera must involve screening out HIV and other potentially serious human pathogens. [P.J. Schwarz and S.S. Arnon, Western J. Med. 156:197 (1992).]

5 Infant botulism has been implicated as the cause of mortality in some cases of Sudden Infant Death Syndrome (SIDS, also known as crib death). SIDS is officially  
10 recognized as infant death that is sudden and unexpected and that remained unexplained despite complete post-mortem examination. The link of SIDS to infant botulism came when fecal or blood specimens taken at autopsy from SIDS infants were found to contain *C. botulinum* organisms and/or toxin in 3-4% of cases analyzed. [D.R. Peterson *et al.*, Rev. Infect. Dis. 1:630 (1979).] In contrast, only 1 of 160 healthy infants (0.6%) had *C. botulinum* organisms in the feces and no botulinal toxin. (S. Arnon *et al.*, Lancet, pp. 1273-76, June 17, 1978.)

15 In developed countries, SIDS is the number one cause of death in children between one month and one year old. (S. Arnon *et al.*, Lancet, pp. 1273-77, June 17, 1978.) More children die from SIDS in the first year than from any other single cause of death in the first fourteen years of life. In the United States, there are 8,000-10,000 SIDS victims annually. *Id.*

20 What is needed is an effective therapy against infant botulism that is free of dangerous side effects, is available in large supply at a reasonable price, and can be safely and gently delivered so that prophylactic application to infants is feasible.

25 Immunization of subjects with toxin preparations has been done in an attempt to induce immunity against botulinal toxins. A *C. botulinum* vaccine comprising chemically inactivated (*i.e.*, formaldehyde-treated) type A, B, C, D and E toxin is commercially available for human usage. However, this vaccine preparation has several disadvantages. First, the efficacy of this vaccine is variable (in particular, only 78% of recipients produce protective levels of anti-type B antibodies following

administration of the primary series). Second, immunization is painful (deep subcutaneous inoculation is required for administration), with adverse reactions being common (moderate to severe local reactions occur in approximately 6% of recipients upon initial injection; this number rises to approximately 11% of individuals who receive booster injections) [Informational Brochure for the Pentavalent (ABCDE) Botulinum Toxoid, Centers for Disease Control]. Third, preparation of the vaccine is dangerous as active toxin must be handled by laboratory workers.

What is needed are safe and effective vaccine preparations for administration to those at risk of exposure to *C. botulinum* toxins.

### *C. difficile*

*C. difficile*, an organism which gained its name due to difficulties encountered in its isolation, has recently been proven to be an etiologic agent of diarrheal disease. (Sneath *et al.*, p. 1165.). *C. difficile* is present in the gastrointestinal tract of approximately 3% of healthy adults, and 10-30% of neonates without adverse effect (Swartz, at p. 644); by other estimates, *C. difficile* is a part of the normal gastrointestinal flora of 2-10% of humans. [G.F. Brooks *et al.*, (eds.) "*Infections Caused by Anaerobic Bacteria*," Jawetz, Melnick, & Adelberg's *Medical Microbiology*, 19th ed., pp. 257-262, Appleton & Lange, San Mateo, CA (1991).] As these organisms are relatively resistant to most commonly used antimicrobials, when a patient is treated with antibiotics, the other members of the normal gastrointestinal flora are suppressed and *C. difficile* flourishes, producing cytopathic toxins and enterotoxins. It has been found in 25% of cases of moderate diarrhea resulting from treatment with antibiotics, especially the cephalosporins, clindamycin, and ampicillin. [M.N. Swartz at 644.]

Importantly, *C. difficile* is commonly associated with nosocomial infections. The organism is often present in the hospital and nursing home environments and may

be carried on the hands and clothing of hospital personnel who care for debilitated and immunocompromised patients. As many of these patients are being treated with antimicrobials or other chemotherapeutic agents, such transmission of *C. difficile* represents a significant risk factor for disease. (Engelkirk *et al.*, pp. 64-67.)

5           *C. difficile* is associated with a range of diarrhetic illness, ranging from diarrhea alone to marked diarrhea and necrosis of the gastrointestinal mucosa with the accumulation of inflammatory cells and fibrin, which forms a pseudomembrane in the affected area. (Brooks *et al.*) It has been found in over 95% of pseudomembranous enterocolitis cases. (Swartz, at p. 644.) This occasionally fatal disease is characterized  
10 by diarrhea, multiple small colonic plaques, and toxic megacolon. (Swartz, at p. 644.) Although stool cultures are sometimes used for diagnosis, diagnosis is best made by detection of the heat labile toxins present in fecal filtrates from patients with enterocolitis due to *C. difficile*. (Swartz, at p. 644-645; and Brooks *et al.*, at p. 260.)  
15 *C. difficile* toxins are cytotoxic for tissue/cell cultures and cause enterocolitis when injected intracecally into hamsters. (Swartz, at p. 644.)

          The enterotoxicity of *C. difficile* is primarily due to the action of two toxins, designated A and B, each of approximately 300,000 in molecular weight. Both are potent cytotoxins, with toxin A possessing direct enterocytotoxic activity. [Lyerly *et al.*, Infect. Immun. 60:4633 (1992).] Unlike toxin A of *C. perfringens*, an organism  
20 rarely associated with antimicrobial-associated diarrhea, the toxin of *C. difficile* is not a spore coat constituent and is not produced during sporulation. (Swartz, at p. 644.) *C. difficile* toxin A causes hemorrhage, fluid accumulation and mucosal damage in rabbit ileal loops and appears to increase the uptake of toxin B by the intestinal mucosa. Toxin B does not cause intestinal fluid accumulation, but it is 1000 times  
25 more toxic than toxin A to tissue culture cells and causes membrane damage. Although both toxins induce similar cellular effects such as actin disaggregation, differences in cell specificity occurs.

Both toxins are important in disease. [Borriello *et al.*, Rev. Infect. Dis., 12(suppl. 2):S185 (1990); Lyster *et al.*, Infect. Immun., 47:349 (1985); and Rolfe, Infect. Immun., 59:1223 (1990).] Toxin A is thought to act first by binding to brush border receptors, destroying the outer mucosal layer, then allowing toxin B to gain access to the underlying tissue. These steps in pathogenesis would indicate that the production of neutralizing antibodies against toxin A may be sufficient in the prophylactic therapy of CDAD. However, antibodies against toxin B may be a necessary additional component for an effective therapeutic against later stage colonic disease. Indeed, it has been reported that animals require antibodies to both toxin A and toxin B to be completely protected against the disease. [Kim and Rolfe, Abstr. Ann. Meet. Am. Soc. Microbiol., 69:62 (1987).]

*C. difficile* has also been reported to produce other toxins such as an enterotoxin different from toxins A and B [Banno *et al.*, Rev. Infect. Dis., 6(Suppl. 1):S11-S20 (1984)], a low molecular weight toxin [Rihn *et al.*, Biochem. Biophys. Res. Comm., 124:690-695 (1984)], a motility altering factor [Justus *et al.*, Gastroenterol., 83:836-843 (1982)], and perhaps other toxins. Regardless, *C. difficile* gastrointestinal disease is of primary concern.

It is significant that due to its resistance to most commonly used antimicrobials, *C. difficile* is associated with antimicrobial therapy with virtually all antimicrobial agents (although most commonly ampicillin, clindamycin and cephalosporins). It is also associated with disease in patients undergoing chemotherapy with such compounds as methotrexate, 5-fluorouracil, cyclophosphamide, and doxorubicin. [S.M. Finegold *et al.*, *Clinical Guide to Anaerobic Infections*, pp. 88-89, Star Publishing Co., Belmont, CA (1992).]

Treatment of *C. difficile* disease is problematic, given the high resistance of the organism. Oral metronidazole, bacitracin and vancomycin have been reported to be effective. (Finegold *et al.*, p. 89.) However there are problems associated with

treatment utilizing these compounds. Vancomycin is very expensive, some patients are unable to take oral medication, and the relapse rate is high (20-25%), although it may not occur for several weeks. *Id.*

5        *C. difficile* disease would be prevented or treated by neutralizing the effects of these toxins in the gastrointestinal tract. Thus, what is needed is an effective therapy against *C. difficile* toxin that is free of dangerous side effects, is available in large supply at a reasonable price, and can be safely delivered so that prophylactic application to patients at risk of developing pseudomembranous enterocolitis can be effectively treated.

## 10        **DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the reactivity of anti-*C. botulinum* IgY by Western blot.

Figure 2 shows the IgY antibody titer to *C. botulinum* type A toxoid in eggs, measured by ELISA.

Figure 3 shows the results of *C. difficile* toxin A neutralization assays.

15        Figure 4 shows the results of *C. difficile* toxin B neutralization assays.

Figure 5 shows the results of *C. difficile* toxin B neutralization assays.

Figure 6 is a restriction map of *C. difficile* toxin A gene, showing sequences of primers 1-4 (SEQ ID NOS:1-4).

Figure 7 is a Western blot of *C. difficile* toxin A reactive protein.

20        Figure 8 shows *C. difficile* toxin A expression constructs.

Figure 9 shows *C. difficile* toxin A expression constructs.

Figure 10 shows the purification of recombinant *C. difficile* toxin A.

Figure 11 shows the results of *C. difficile* toxin A neutralization assays with antibodies reactive to recombinant toxin A.

25        Figure 12 shows the results for a *C. difficile* toxin A neutralization plate.

Figure 13 shows the results for a *C. difficile* toxin A neutralization plate.



Figure 14 shows the results of recombinant *C. difficile* toxin A neutralization assays.

Figure 15 shows *C. difficile* toxin A expression constructs.

5 Figure 16 shows a chromatograph plotting absorbance at 280 nm against retention time for a pMA1870-680 IgY PEG preparation.

Figure 17 shows two recombinant *C. difficile* toxin B expression constructs.

Figure 18 shows *C. difficile* toxin B expression constructs.

Figure 19 shows *C. difficile* toxin B expression constructs.

Figure 20 shows *C. difficile* toxin B expression constructs.

10 Figure 21 is an SDS-PAGE gel showing the purification of recombinant *C. difficile* toxin B fusion protein.

Figure 22 is an SDS-PAGE gel showing the purification of two histidine-tagged recombinant *C. difficile* toxin B proteins.

Figure 23 shows *C. difficile* toxin B expression constructs.

15 Figure 24 is a Western blot of *C. difficile* toxin B reactive protein.

Figure 25 shows *C. botulinum* type A toxin expression constructs; constructs used to provide *C. botulinum* or *C. difficile* sequences are also shown.

Figure 26 is an SDS-PAGE gel stained with Coomassie blue showing the purification of recombinant *C. botulinum* type A toxin fusion proteins.

20 Figure 27 shows *C. botulinum* type A toxin expression constructs; constructs used to provide *C. botulinum* sequences are also shown.

Figure 28 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBot protein using the Ni-NTA resin.

25 Figure 29 is an SDS-PAGE gel stained with Coomassie blue showing the expression of pHisBot protein in BL21(DE3) and BL21(DE3)pLysS host cells.

Figure 30 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBot protein using a batch absorption procedure.

Figure 31 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBot and pHisBot(native) proteins using a Ni-NTA column.

Figure 32 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBotA protein expressed in pHisBotA(syn) kan lacIq

5 T7/pACYCGro/BL21(DE3) cells using an IDA column.

Figure 33 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBotA, pHisBotB and pHisBotE proteins by IDA chromatography followed by chromatography on S-100 to remove folding chaperones.

10 Figure 34 is an SDS-PAGE gel stained with Coomassie blue showing the extracts derived from pHisBotB amp T7lac/BL21(DE3) cells before and after purification on a Ni-NTA column.

Figure 35 is an SDS-PAGE gel run under native conditions and stained with Coomassie blue showing the removal of folding chaperones from IDA-purified BotB protein using a S-100 column.

15 Figure 36 is an SDS-PAGE gel stained with Coomassie blue showing proteins that eluted during an imidazole step gradient applied to a IDA column containing a lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells.

Figure 37 is an SDS-PAGE gel run under native conditions and stained with Coomassie blue showing IDA-purified BotB protein before and after ultrafiltration.

20 Figure 38 is an SDS-PAGE gel stained with Coomassie blue showing the purification of BotE protein using a NiNTA column.

Figure 39 is an SDS-PAGE gel stained with Coomassie blue showing extracts derived from pHisBotA kan T7 lac/BL21(DE3) pLysS cells grown in fermentation culture.

25 Figure 40 is a chromatogram showing proteins present after IDA-purified BotE protein was applied to a S-100 column.

## DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

5 As used herein, the term "neutralizing" is used in reference to antitoxins, particularly antitoxins comprising antibodies, which have the ability to prevent the pathological actions of the toxin against which the antitoxin is directed.

10 As used herein, the term "overproducing" is used in reference to the production of clostridial toxin polypeptides in a host cell and indicates that the host cell is producing more of the clostridial toxin by virtue of the introduction of nucleic acid sequences encoding said clostridial toxin polypeptide than would be expressed by said host cell absent the introduction of said nucleic acid sequences. To allow ease of purification of toxin polypeptides produced in a host cell it is preferred that the host cell express or overproduce said toxin polypeptide at a level greater than 1 mg/liter of host cell culture.

15 "A host cell capable of expressing a recombinant protein at a level greater than or equal to 5% of the total cellular protein" is a host cell in which the recombinant protein represents at least 5% of the total cellular protein. To determine what percentage of total cellular protein the recombinant protein represents, the following steps are taken. A total of 10 OD<sub>600</sub> units of recombinant host cells (*e.g.*, 200 µl of cells at OD<sub>600</sub>=50/ml) are removed (at a timepoint known to represent the peak of expression of the desired recombinant protein) to a 1.5 ml microfuge tube and pelleted for 2 min at maximum rpm in a microfuge. The pellets are resuspended in 1 ml of 50 mM NaHPO<sub>4</sub>, 0.5 M NaCl, 40 mM imidazole buffer (pH 6.8) containing 1 mg/ml lysozyme. The samples are incubated for 20 min at room temperature and stored ON  
20 at -70°C. Samples are thawed completely at room temperature and sonicated 2 X 10 seconds with a Branson Sonifier 450 microtip probe at # 3 power setting. The  
25 samples are centrifuged for 5 min. at maximum rpm in a microfuge. An aliquot (20

μl) of the protein sample is removed to 20 μl 2X sample buffer (this represents the total protein extract). The samples are heated to 95°C for 5 min, then cooled and 5 or 10 μl are loaded onto 12.5% SDS-PAGE gels. High molecular weight protein markers are also loaded to allow for estimation of the MW of identified recombinant proteins.

5 After electrophoresis, protein is detected generally by staining with Coomassie blue and the stained gel is scanned using a densitometer to determine the percentage of protein present in each band. In this manner, the percentage of protein present in the band corresponding to the recombinant protein of interest may be determined. It is not necessary that Coomassie blue be employed for the detection of protein, a number of  
10 fluorescent dyes [*e.g.*, Sypro orange S-6651 (Molecular Probes, Eugene, OR)] may be employed and the stained gel scanned using a fluoroiager [*e.g.*, Fluor Imager SI (Molecular Dynamics, Sunnyvale, CA)].

"A host cell capable of expressing a recombinant protein as a soluble protein at a level greater than or equal to 0.25% of the total soluble cellular protein" is a host  
15 cell in which the amount of soluble recombinant protein present represents at least 0.25% of the total cellular protein. As used herein "total soluble cellular protein" refers to a clarified PEI lysate prepared as described in Example 31(c)(iv). Briefly, cells are harvested following induction of expression of recombinant protein (at a point of maximal expression). The cells are resuspended in cell resuspension buffer (CRB:  
20 50 mM NaPO<sub>4</sub>, 0.5 M NaCl, 40 mM imidazole, pH 6.8) to create a 20% cell suspension (wet weight of cells/volume of CRB) and cell lysates are prepared as described in Example 31(c)(iv) (*i.e.*, sonication or homogenization followed by centrifugation). The cell lysate is then flocculated utilizing polyethyleneimine (PEI) prior to centrifugation. PEI (a 2% solution in dH<sub>2</sub>O, pH 7.5 with HCl) is added to the  
25 cell lysate to a final concentration of 0.2%, and stirred for 20 min at room temperature prior to centrifugation [8,500 rpm in JA10 rotor (Beckman) for 30 minutes at 4°C]. This treatment removes RNA, DNA and cell wall components, resulting in a clarified,

low viscosity lysate ("PEI clarified lysate"). The recombinant protein present in the PEI clarified lysate is then purified (e.g., by chromatography on an IDA column for his-tagged proteins). The amount of purified recombinant protein (i.e., the eluted protein) is divided by the concentration of protein present in the PEI clarified lysate (typically 8 mg/ml when using a 20% cell suspension as the starting material) and multiplied by 100 to determine what percentage of total soluble cellular protein is comprised of the soluble recombinant protein (see Example 33b).

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (i.e., *C. botulinum* toxin A, B, C, D, E, F, or G and fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the *C. botulinum* protein as expressed in a host cell, may provide an affinity tag to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (i.e., toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

As used herein the term "non-toxin protein" or "non-toxin protein sequence" refers to that portion of a fusion protein which comprises a protein or protein sequence which is not derived from a bacterial toxin protein.

The term "protein of interest" as used herein refers to the protein whose expression is desired within the fusion protein. In a fusion protein the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

As used herein, the term "maltose binding protein" refers to the maltose binding protein of *E. coli*. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein; a portion of the maltose binding protein may

merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

5 As used herein, the term "poly-histidine tract" when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate or IDA  
10 column.

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind toxin. The removal of non-immunoglobulin  
15 proteins and/or the removal of immunoglobulins that do not bind toxin results in an increase in the percent of toxin-reactive immunoglobulins in the sample. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified by the removal of host cell proteins; the percent of recombinant toxin polypeptides is thereby increased in the sample. Additionally, the recombinant toxin polypeptides are purified by the removal of host cell components  
20 such as lipopolysaccharide (*e.g.*, endotoxin).

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

25 The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

The term "native protein" as used herein refers to a protein which is isolated from a natural source as opposed to the production of a protein by recombinant means.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell is a protein which exists in solution in the cytoplasm of the host cell; if the protein contains a signal sequence the soluble protein is exported to the periplasmic space in bacteria hosts and is secreted into the culture medium in eucaryotic cells capable of secretion or by bacterial host possessing the appropriate genes (*i.e.*, the *kil* gene). In contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called inclusion bodies) in the host cell. High level expression (*i.e.*, greater than 10-20 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble protein is a protein which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

A distinction is drawn between a soluble protein (*i.e.*, a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble recombinant protein found inside an inclusion body may be solubilized (*i.e.*, rendered into a soluble form) by treating purified inclusion bodies with denaturants such as guanidine hydrochloride, urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a denaturant and removal of the denaturant. Many

proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and will maintain the proteins in solution at low concentration. However, dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out); therefore, SDS-solubilized inclusion body protein is soluble but not refolded.

A distinction is drawn between proteins which are soluble (*i.e.*, dissolved) in a solution devoid of significant amounts of ionic detergents (*e.g.*, SDS) or denaturants (*e.g.*, urea, guanidine hydrochloride) and proteins which exist as a suspension of insoluble protein molecules dispersed within the solution. A soluble protein will not be removed from a solution containing the protein by centrifugation using conditions sufficient to remove bacteria present in a liquid medium (*i.e.*, centrifugation at 12,000 x g for 4-5 minutes). For example, to test whether two proteins, protein A and protein B, are soluble in solution, the two proteins are placed into a solution selected from the group consisting of PBS-NaCl (PBS containing 0.5 M NaCl), PBS-NaCl containing 0.2% Tween 20, PBS, PBS containing 0.2% Tween 20, PBS-C (PBS containing 2 mM CaCl<sub>2</sub>), PBS-C containing either 0.1 or 0.5 % Tween 20, PBS-C containing either 0.1 or 0.5% NP-40, PBS-C containing either 0.1 or 0.5% Triton X-100, PBS-C containing 0.1% sodium deoxycholate. The mixture containing proteins A and B is then centrifuged at 5000 x g for 5 minutes. The supernatant and pellet formed by centrifugation are then assayed for the presence of protein A and B. If protein A is found in the supernatant and not in the pellet [except for minor amounts (*i.e.*, less than 10%) as a result of trapping], protein is said to be soluble in the solution tested. If the majority of protein B is found in the pellet (*i.e.*, greater than 90%), then protein B is said to exist as a suspension in the solution tested.

As used herein, the term "therapeutic amount" refers to that amount of antitoxin required to neutralize the pathologic effects of one or more clostridial toxins in a subject.



The term "pyrogen" as used herein refers to a fever-producing substance. Pyrogens may be endogenous to the host (*e.g.*, prostaglandins) or may be exogenous compounds (*e.g.*, bacterial endo- and exotoxins, nonbacterial compounds such as antigens and certain steroid compounds, etc.). The presence of pyrogen in a pharmaceutical solution may be detected using the U.S. Pharmacopeia (USP) rabbit fever test (United States Pharmacopeia, Vol. XXII (1990) United States Pharmacopeial Convention, Rockville, MD, p. 151).

The term "endotoxin" as used herein refers to the high molecular weight complexes associated with the outer membrane of gram-negative bacteria. Unpurified endotoxin contains lipids, proteins and carbohydrates. Highly purified endotoxin does not contain protein and is referred to as lipopolysaccharide (LPS). Because unpurified endotoxin is of concern in the production of pharmaceutical compounds (*e.g.*, proteins produced in *E. coli* using recombinant DNA technology), the term endotoxin as used herein refers to unpurified endotoxin. Bacterial endotoxin is a well known pyrogen.

As used herein, the term "endotoxin-free" when used in reference to a composition to be administered parenterally (with the exception of intrathecal administration) to a host means that the dose to be delivered contains less than 5 EU/kg body weight [FDA Guidelines for Parenteral Drugs (December 1987)]. Assuming a weight of 70 kg for an adult human, the dose must contain less than 350 EU to meet FDA Guidelines for parenteral administration. Endotoxin levels are measured herein using the Limulus Amebocyte Lysate (LAL) test (Limulus Amebocyte Lysate Pyrochrome™, Associates of Cape Cod, Inc. Woods Hole, MA). To measure endotoxin levels in preparations of recombinant proteins, 0.5 ml of a solution comprising 0.5 mg of purified recombinant protein in 50 mM NaPO<sub>4</sub>, pH 7.0, 0.3M NaCl and 10% glycerol is used in the LAL assay according to the manufacturer's instructions for the endpoint chromogenic without diazo-coupling method [the specific components of the buffer containing recombinant protein to be analyzed in the LAL

test are not important; any buffer having a neutral pH may be employed (see for example, alternative buffers employed in Examples 34, 40 and 45)]. Compositions containing less than or equal to than 250 endotoxin units (EU)/mg of purified recombinant protein are herein defined as "substantially endotoxin-free." Preferably the composition contains less than or equal to 100, and most preferably less than or equal to 60, (EU)/mg of purified recombinant protein. Typically, administration of bacterial toxins or toxoids to adult humans for the purpose of vaccination involves doses of about 10-500 µg protein/dose. Therefore, administration of 10-500 µg of a purified recombinant protein to a 70 kg human, wherein said purified recombinant protein preparation contains 60 EU/mg protein, results in the introduction of only 0.6 to 30 EU (*i.e.*, 0.2 to 8.6% of the maximum allowable endotoxin burden per parenteral dose). Administration of 10-500 µg of a purified recombinant protein to a 70 kg human, wherein said purified recombinant protein preparation contains 250 EU/mg protein, results in the introduction of only 2.5 to 125 EU (*i.e.*, 0.7 to 36% of the maximum allowable endotoxin burden per parenteral dose).

The LAL test is accepted by the U.S. FDA as a means of detecting bacterial endotoxins (21 C.F.R. §§ 660.100 -105). Studies have shown that the LAL test is equivalent or superior to the USP rabbit pyrogen test for the detection of endotoxin and thus the LAL test can be used as a surrogate for pyrogenicity studies in animals [F.C. Perason, *Pyrogens: endotoxins, LAL testing and depyrogenation*, Marcel Dekker, New York (1985), pp.150-155]. The FDA Bureau of Biologics accepts the LAL assay in place of the USP rabbit pyrogen test so long as the LAL assay utilized is shown to be as sensitive as, or more sensitive as the rabbit test [Fed. Reg., 38, 26130 (1980)].

The term "monovalent" when used in reference to a clostridial vaccine refers to a vaccine which is capable of provoking an immune response in a host animal directed against a single type of clostridial toxin. For example, if immunization of a host with *C. botulinum* type A toxin vaccine induces antibodies in the immunized host which

protect against a challenge with type A toxin but not against challenge with type B, C, D, E, F or G toxins, then the type A vaccine is said to be monovalent. In contrast, a "multivalent" vaccine provokes an immune response in a host animal directed against several (*i.e.*, more than one) clostridial toxins. For example, if immunization of a host with a vaccine comprising *C. botulinum* type A and B toxins induces the production of antibodies which protect the host against a challenge with both type A and B toxin, the vaccine is said to be multivalent (in particular, this hypothetical vaccine is bivalent).

As used herein the term "immunogenically-effective amount" refers to that amount of an immunogen required to invoke the production of protective levels of antibodies in a host upon vaccination.

The term "protective level", when used in reference to the level of antibodies induced upon immunization of the host with an immunogen which comprises a bacterial toxin, means a level of circulating antibodies sufficient to protect the host from challenge with a lethal dose of the toxin.

As used herein the terms "protein" and "polypeptide" refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably.

The terms "toxin" and "neurotoxin" when used in reference to toxins produced by members (*i.e.*, species and strains) of the genus *Clostridium* are used interchangeably and refer to the proteins which are poisonous to nerve tissue.

The term "receptor-binding domain" when used in reference to a *C. botulinum* toxin refers to the carboxy-terminal portion of the heavy chain ( $H_C$  or the C fragment) of the toxin which is presumed to be responsible for the binding of the active toxin (*i.e.*, the derivative toxin comprising the H and L chains joined via disulfide bonds) to receptors on the surface of synaptosomes. The receptor-binding domain for *C.*

*botulinum* type A toxin is defined herein as comprising amino acid residues 861 through 1296 of SEQ ID NO:28. The receptor-binding domain for *C. botulinum* type B toxin is defined herein as comprising amino acid residues 848 through 1291 of SEQ

ID NO:40 (strain Eklund 17B). The receptor-binding domain of *C. botulinum* type C1 toxin is defined herein as comprising amino acid residues 856 through 1291 of SEQ ID NO:60. The receptor-binding domain of *C. botulinum* type D toxin is defined herein as comprising amino acid residues 852 through 1276 of SEQ ID NO:66. The  
5 receptor-binding domain of *C. botulinum* type E toxin is defined herein as comprising amino acid residues 835 through 1250 of SEQ ID NO:50 (Beluga strain). The receptor-binding domain of *C. botulinum* type F toxin is defined herein as comprising amino acid residues 853 through 1274 of SEQ ID NO:71. The receptor-binding domain of *C. botulinum* type G toxin is defined herein as comprising amino acid  
10 residues 853 through 1297 of SEQ ID NO:77. Within a given serotype, small variations in the primary amino acid sequence of the botulinal toxins isolated from different strains has been reported [Whelan *et al.* (1992), *supra* and Minton (1995) Curr. Top. Microbiol. Immunol. 195:161-194]. The present invention contemplates fusion proteins comprising the receptor-binding domain of *C. botulinum* toxins from  
15 serotypes A-G including the variants found among different strains within a given serotype. The receptor-binding domains listed above are used as the prototype for each strain within a serotype. Fusion proteins containing an analogous region from a strain other than the prototype strain are encompassed by the present invention.

Fusion proteins comprising the receptor binding domain (*i.e.*, C fragment) of  
20 botulinal toxins may include amino acid residues located beyond the termini of the domains defined above. For example, the pHisBotB protein contains amino acid residues 846-1291 of SEQ ID NO:40; this fusion protein thus comprises the receptor-binding domain for *C. botulinum* type B toxin as defined above (*i.e.*, Ile-848 through Glu-1291). Similarly, pHisBotE contains amino acid residues 827-1252 of SEQ ID  
25 NO:50 and pHisBotG contains amino acid residues 851-1297 of SEQ ID NO:77. Thus, both pHisBotE and pHisBotG fusion proteins contain a few amino acids located beyond the N-terminus of the defined receptor-binding domain.

The terms "native gene" or "native gene sequences" are used to indicate DNA sequences encoding a particular gene which contain the same DNA sequences as found in the gene as isolated from nature. In contrast, "synthetic gene sequences" are DNA sequences which are used to replace the naturally occurring DNA sequences when the naturally occurring sequences cause expression problems in a given host cell. For example, naturally-occurring DNA sequences encoding codons which are rarely used in a host cell may be replaced (*e.g.*, by site-directed mutagenesis) such that the synthetic DNA sequence represents a more frequently used codon. The native DNA sequence and the synthetic DNA sequence will preferably encode the same amino acid sequence.

## SUMMARY OF THE INVENTION

The present invention relates to the production of polypeptides derived from toxins particularly in recombinant host cells. In one embodiment, the present invention provides a host cell containing a recombinant expression vector, said vector encoding a protein comprising at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The present invention is not limited by the nature of sequences encoding portions of the *C. botulinum* toxin. These sequences may be derived from the native gene sequences or alternatively they may comprise synthetic gene sequences. Synthetic gene sequences are employed when expression of the native gene sequences is problematic in a given host cell (*e.g.*, when the native gene sequences contain sequences resembling yeast transcription termination signals and the desired host cell is a yeast cell).

In one embodiment, the host cell is capable of expressing the recombinant *C. botulinum* toxin protein at a level greater than or equal to 2% to 40% of the total cellular protein and preferably at a level greater than or equal to 5% of the total cellular protein. In another embodiment, the host cell is capable of expressing the recombinant *C. botulinum* toxin protein as a soluble protein at a level greater than or

equal to 0.25% of the total cellular protein and preferably at a level greater than or equal to 0.25% to 10% of the total cellular protein.

The present invention is not limited by the nature of the host cell employed for the production of recombinant *C. botulinum* toxin proteins. In a preferred  
5 embodiment, the host cell is an *E. coli* cell. In another preferred embodiment, the host cell is an insect cell; particularly preferred insect host cells are *Spodoptera frugiperda* (Sf9) cells. In another preferred embodiment, the host cell is a yeast cell; particularly preferred yeast cells are *Pichia pastoris* cells.

In another embodiment, the invention provides a host cell containing a  
10 recombinant expression vector, said vector encoding a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The invention is not limited by the nature of the portion of the *Clostridium botulinum* toxin selected. In a preferred embodiment, the portion of the toxin comprises the receptor  
15 binding domain (*i.e.*, the C fragment of the toxin). The present invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (*e.g.*, MBP, GST, protein A, etc.) and may be employed for the production of fusion proteins  
20 comprising a portion of a botulinal toxin.

The present invention further provides a vaccine comprising a fusion protein, said fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The vaccine may be a monovalent vaccine (*i.e.*, containing  
25 only a toxin B fusion protein or a toxin E fusion protein), a bivalent vaccine (*i.e.*, containing both a toxin B fusion protein and a toxin E fusion protein) or a trivalent or higher valency vaccine. In a preferred embodiment, the toxin B fusion protein and/or

toxin E fusion protein is combined with a fusion protein comprising a non-toxin protein sequence and at least a portion of *Clostridium botulinum* type A toxin. The present invention is not limited by the nature of the portion of the *Clostridium botulinum* toxin selected. In a preferred embodiment, the portion of the toxin  
5 comprises the receptor binding domain (*i.e.*, the C fragment of the toxin). The present invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (*e.g.*, MBP, GST, protein A, etc.) and may be employed for the generation of fusion  
10 proteins comprising vaccines. When a fusion partner (*i.e.*, the non-toxin protein sequence) is employed for the production of a recombinant *C. botulinum* toxin protein, the fusion partner may be removed from the recombinant *C. botulinum* toxin protein if desired (*i.e.*, prior to administration of the protein to a subject) using a variety of methods known to the art (*e.g.*, digestion of fusion proteins containing FactorXa or  
15 thrombin recognition sites with the appropriate enzyme). A number of the pETHis vectors employed herein provide an N-terminal his-tag followed by a FactorXa cleavage site (see Example 28a); the botulinum C fragment sequences follow the FactorXa site and thus, FactorXa can be used to remove the his-tag from the botulinum fusion protein. In a preferred embodiment, the vaccine is substantially endotoxin-free.

20 The present invention is not limited by the method employed for the generation of vaccine comprising fusion proteins comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin. The fusion proteins may be produced by recombinant DNA means using either native or synthetic gene sequences expressed in a host cell. The present invention is not limited to the production of vaccines using  
25 recombinant host cells; cell free *in vitro* transcription/translation systems may be employed for the expression of the nucleic acid constructs encoding the fusion proteins of the present invention. An example of such a cell-free system is the commercially

available TnT™ Coupled Reticulocyte Lysate System (Promega Corporation, Madison, WI). Alternatively, the fusion proteins of the present invention may be generated by synthetic means (*i.e.*, peptide synthesis).

5 The present invention further provides a method of generating antibody directed against a *Clostridium botulinum* toxin comprising: a) providing in any order: i) an antigen comprising a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin, and ii) a host; and b) immunizing the host with the antigen so as to generate an antibody. In a preferred embodiment, the antigen  
10 used to immunize the host also contains a fusion protein comprising a non-toxin protein sequence and at least a portion of *Clostridium botulinum* type A toxin. The present invention is not limited by the nature of the portion of the *Clostridium botulinum* toxin selected. In a preferred embodiment, the portion of the toxin comprises the receptor binding domain (*i.e.*, the C fragment of the toxin). The present  
15 invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (*e.g.*, MBP, GST, protein A, etc.) and may be employed for the generation of fusion proteins comprising vaccines. When a fusion partner (*i.e.*, the non-toxin protein  
20 sequence) is employed for the production of a recombinant *C. botulinum* toxin protein, the fusion partner may be removed from the recombinant *C. botulinum* toxin protein if desired (*i.e.*, prior to administration of the protein to a subject) using a variety of methods known to the art (*e.g.*, digestion of fusion proteins containing FactorXa or thrombin recognition sites with the appropriate enzyme).

25 The present invention is not limited by the nature of the host employed for the production of the antibodies of the invention. In a preferred embodiment, the host is a mammal, preferably a human. The antibodies of the present invention may be



generated using non-mammalian hosts such as birds, preferably chickens. In a preferred embodiment the method of the present invention further comprised the step c) of collecting the antibodies from the host. In yet another embodiment, the method of the present invention further comprises the step d) of purifying the antibodies.

5       The present invention further provides antibodies raised according to the above methods.

10       The present invention further contemplates multivalent vaccines comprising at least two recombinant *C. botulinum* toxin proteins derived from the group consisting of *C. botulinum* serotypes A, B, C, D, E, F, and G. The invention contemplates bivalent, trivalent, quadravalent, pentavalent, heptavalent and septivalent vaccines comprising recombinant *C. botulinum* toxin proteins. Preferably the recombinant *C. botulinum* toxin protein comprises the receptor binding domain (*i.e.*, C fragment) of the toxin.

## DESCRIPTION OF THE INVENTION

15       The present invention contemplates vaccinating humans and other animals with polypeptides derived from *C. botulinum* neurotoxins which are substantially endotoxin-free. These botulinal peptides are also useful for the production of antitoxin. Anti-botulinal toxin antitoxin is useful for the treatment of patients effected by or at risk of symptoms due to the action of *C. botulinum* toxins. The organisms, toxins and individual steps of the present invention are described separately below.

### 20    I.    *Clostridium* Species, Clostridial Diseases And Associated Toxins

25       A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against *Clostridium* species, their toxins, enzymes or other metabolic by-products, cell wall components, or synthetic or recombinant versions of any of these compounds. It is contemplated that these antibodies will be produced by immunization of humans or other animals. It is not intended that the present invention

be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *Clostridium* species are contemplated as immunogens. Examples of these toxins include the neuraminidase toxin of *C. butyricum*, *C. sordellii* toxins HT and LT, toxins A, B, C, D, E, F, and G of *C. botulinum* and the numerous *C.*

*perfringens* toxins. In one preferred embodiment, toxins A, B, and E of *C. botulinum* are contemplated as immunogens. Table 2 above lists various *Clostridium* species, their toxins and some antigens associated with disease.

TABLE 2

Clostridial Toxins

Organism	Toxins and Disease-Associated Antigens
<i>C. botulinum</i>	A, B, C <sub>1</sub> , C <sub>2</sub> , D, E, F, G
<i>C. butyricum</i>	Neuraminidase
<i>C. difficile</i>	A, B, Enterotoxin (not A nor B), Motility Altering Factor, Low Molecular Weight Toxin, Others
<i>C. perfringens</i>	$\alpha$ , $\beta$ , $\epsilon$ , $\iota$ , $\gamma$ , $\delta$ , $\nu$ , $\theta$ , $\kappa$ , $\lambda$ , $\mu$ , $\upsilon$
<i>C. sordellii</i> / <i>C. bifermentans</i>	HT, LT, $\alpha$ , $\beta$ , $\gamma$
<i>C. novyi</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$ , $\epsilon$ , $\zeta$ , $\nu$ , $\theta$
<i>C. septicum</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$
<i>C. histolyticum</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$ , $\epsilon$ plus additional enzymes
<i>C. chauvoei</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$

It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin (e.g., *C. perfringens* type A enterotoxin) may be used as an effective therapeutic against one or more toxin(s) produced by other members of the genus *Clostridium* or other toxin producing organisms (e.g., *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus mutans*, *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa*, other *Pseudomonas*

species, etc.). It is further contemplated that antibodies directed against the portion of the toxin which binds to mammalian membranes (e.g., *C. perfringens* enterotoxin A) can also be used against other organisms. It is contemplated that these membrane binding domains are produced synthetically and used as immunogens.

## 5        II.        Obtaining Antibodies In Non-Mammals

A preferred embodiment of the method of the present invention for obtaining antibodies involves immunization. However, it is also contemplated that antibodies could be obtained from non-mammals without immunization. In the case where no immunization is contemplated, the present invention may use non-mammals with  
10        preexisting antibodies to toxins as well as non-mammals that have antibodies to whole organisms by virtue of reactions with the administered antigen. An example of the latter involves immunization with synthetic peptides or recombinant proteins sharing epitopes with whole organism components.

In a preferred embodiment, the method of the present invention contemplates  
15        immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment, toxin from all clostridial bacteria sources (see Table 2) are contemplated as immunogens. Examples of these toxins are *C. butyricum* neuraminidase toxin, toxins A, B, C, D, E, F, and G from *C. botulinum* , *C. perfringens* toxins  $\alpha$ ,  $\beta$ ,  $\epsilon$ , and  $\iota$ , and *C. sordellii* toxins HT and LT. In a preferred embodiment, *C. botulinum* toxins A, B, C, D, E, and F (or  
20        fragments thereof) are contemplated as immunogens.

A particularly preferred embodiment involves the use of bacterial toxin protein or fragments of toxin proteins produced by molecular biological means (i.e., recombinant toxin proteins). In a preferred embodiment, the immunogen comprises  
25        the receptor-binding domain (i.e., the ~50 kD carboxy-terminal portion of the heavy chain; also referred to as the C fragment) of *C. botulinum* serotype A neurotoxin produced by recombinant DNA technology. In another preferred embodiment, the immunogen comprises the receptor-binding domain of *C. botulinum* serotype B

neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype E neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype C1 neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype C2 neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype D neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype F neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype G neurotoxin produced by recombinant DNA technology. In a preferred embodiment, the recombinant botulinal toxin proteins are expressed as fusion proteins (*e.g.*, as histidine-tagged proteins). In a still further preferred embodiment, the immunogen is a multivalent vaccine comprising the receptor-binding domain region of *C. botulinum* toxin from two or more toxins selected from the group consisting of type A, type B, type C (including C1 and C2), type D, type E, and type F toxin.

When immunization is used, the preferred non-mammal is from the class *Aves*. All birds are contemplated (*e.g.*, duck, ostrich, emu, turkey, etc.). A preferred bird is a chicken. Importantly, chicken antibody does not fix mammalian complement. [See H.N. Benson *et al.*, *J. Immunol.* 87:616 (1961).] Thus, chicken antibody will normally not cause a complement-dependent reaction. [A.A. Benedict and K. Yamaga, "*Immunoglobulins and Antibody Production in Avian Species*," in *Comparative Immunology* (J.J. Marchaloni, ed.), pp. 335-375, Blackwell, Oxford (1966).] Thus, the preferred antitoxins of the present invention will not exhibit complement-related side effects observed with antitoxins known presently.

When birds are used, it is contemplated that the antibody will be obtained from either the bird serum or the egg. A preferred embodiment involves collection of the antibody from the egg. Laying hens transport immunoglobulin to the egg yolk ("IgY") in concentrations equal to or exceeding that found in serum. [See R. Patterson *et al.*, J. Immunol. 89:272 (1962); and S.B. Carroll and B.D. Stollar, J. Biol. Chem. 258:24 (1983).] In addition, the large volume of egg yolk produced vastly exceeds the volume of serum that can be safely obtained from the bird over any given time period. Finally, the antibody from eggs is purer and more homogeneous; there is far less non-immunoglobulin protein (as compared to serum) and only one class of immunoglobulin is transported to the yolk.

When considering immunization with toxins, one may consider modification of the toxins to reduce the toxicity. In this regard, it is not intended that the present invention be limited by immunization with modified toxin. Unmodified ("native") toxin is also contemplated as an immunogen.

It is also not intended that the present invention be limited by the type of modification -- if modification is used. The present invention contemplates all types of toxin modification, including chemical and heat treatment of the toxin. The preferred modification, however, is formaldehyde treatment.

It is not intended that the present invention be limited to a particular mode of immunization; the present invention contemplates all modes of immunization, including subcutaneous, intramuscular, intraperitoneal, and intravenous or intravascular injection, as well as *per os* administration of immunogen.

The present invention further contemplates immunization with or without adjuvant. (Adjuvant is defined as a substance known to increase the immune response to other antigens when administered with other antigens.) If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of adjuvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. Another preferred use of adjuvant is the use

of Gerbu Adjuvant. The invention also contemplates the use of RIBI fowl adjuvant and Quil A adjuvant.

When immunization is used, the present invention contemplates a wide variety of immunization schedules. In one embodiment, a chicken is administered toxin(s) on day zero and subsequently receives toxin(s) in intervals thereafter. It is not intended that the present invention be limited by the particular intervals or doses. Similarly, it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is sometime after day 100.

Where birds are used and collection of antibody is performed by collecting eggs, the eggs may be stored prior to processing for antibody. It is preferred that eggs be stored at 4°C for less than one year.

It is contemplated that chicken antibody produced in this manner can be buffer-extracted and used analytically. While unpurified, this preparation can serve as a reference for activity of the antibody prior to further manipulations (*e.g.*, immunoaffinity purification).

### III. Increasing The Effectiveness Of Antibodies

When purification is used, the present invention contemplates purifying to increase the effectiveness of both non-mammalian antitoxins and mammalian antitoxins. Specifically, the present invention contemplates increasing the percent of toxin-reactive immunoglobulin. The preferred purification approach for avian antibody is polyethylene glycol (PEG) separation.

The present invention contemplates that avian antibody be initially purified using simple, inexpensive procedures. In one embodiment, chicken antibody from eggs is purified by extraction and precipitation with PEG. PEG purification exploits the differential solubility of lipids (which are abundant in egg yolks) and yolk proteins in high concentrations of PEG 8000. [Polson *et al.*, Immunol. Comm. 9:495 (1980).] The technique is rapid, simple, and relatively inexpensive and yields an immunoglobulin fraction that is significantly purer in terms of contaminating non-immunoglobulin proteins than the comparable ammonium sulfate fractions of

mammalian sera and horse antibodies. The majority of the PEG is removed from the precipitated chicken immunoglobulin by treatment with ethanol. Indeed, PEG-purified antibody is sufficiently pure that the present invention contemplates the use of PEG-purified antitoxins in the passive immunization of intoxicated humans and animals.

#### 5      **IV.    Treatment**

The present invention contemplates antitoxin therapy for humans and other animals intoxicated by bacterial toxins. A preferred method of treatment is by intravenous administration of anti-boutlinal antitoxin; oral administration is also contemplated for other clostridial antitoxins.

##### 10            **A.      Dosage Of Antitoxin**

It was noted by way of background that a balance must be struck when administering currently available antitoxin which is usually produced in large animals such as horses; sufficient antitoxin must be administered to neutralize the toxin, but not so much antitoxin as to increase the risk of untoward side effects. These side  
15 effects are caused by: i) patient sensitivity to foreign (*e.g.*, horse) proteins; ii) anaphylactic or immunogenic properties of non-immunoglobulin proteins; iii) the complement fixing properties of mammalian antibodies; and/or iv) the overall burden of foreign protein administered. It is extremely difficult to strike this balance when, as noted above, the degree of intoxication (and hence the level of antitoxin therapy  
20 needed) can only be approximated.

The present invention contemplates significantly reducing side effects so that this balance is more easily achieved. Treatment according to the present invention contemplates reducing side effects by using PEG-purified antitoxin from birds.

In one embodiment, the treatment of the present invention contemplates the use  
25 of PEG-purified antitoxin from birds. The use of yolk-derived, PEG-purified antibody as antitoxin allows for the administration of: 1) non(mammalian)-complement-fixing, avian antibody; 2) a less heterogeneous mixture of non-immunoglobulin proteins; and 3) less total protein to deliver the equivalent weight of active antibody present in

currently available antitoxins. The non-mammalian source of the antitoxin makes it useful for treating patients who are sensitive to horse or other mammalian sera.

### **B. Delivery Of Antitoxin**

Although it is not intended to limit the route of delivery, the present invention  
5 contemplates a method for antitoxin treatment of bacterial intoxication in which  
delivery of antitoxin is oral. In one embodiment, antitoxin is delivered in a solid form  
(*e.g.*, tablets). In an alternative embodiment antitoxin is delivered in an aqueous  
solution. When an aqueous solution is used, the solution has sufficient ionic strength  
to solubilize antibody protein, yet is made palatable for oral administration. The  
10 delivery solution may also be buffered (*e.g.*, carbonate buffer pH 9.5) which can  
neutralize stomach acids and stabilize the antibodies when the antibodies are  
administered orally. In one embodiment the delivery solution is an aqueous solution.  
In another embodiment the delivery solution is a nutritional formula. Preferably, the  
delivery solution is infant formula. Yet another embodiment contemplates the delivery  
15 of lyophilized antibody encapsulated or microencapsulated inside acid-resistant  
compounds.

Methods of applying enteric coatings to pharmaceutical compounds are well  
known to the art [companies specializing in the coating of pharmaceutical compounds  
are available; for example, The Coating Place (Verona, WI) and AAI (Wilmington,  
20 NC)]. Enteric coatings which are resistant to gastric fluid and whose release (*i.e.*,  
dissolution of the coating to release the pharmaceutical compound) is pH dependent  
are commercially available [for example, the polymethacrylates Eudragit® L and  
Eudragit® S (Röhm GmbH)]. Eudragit® S is soluble in intestinal fluid from pH 7.0;  
this coating can be used to microencapsulate lyophilized antitoxin antibodies and the  
25 particles are suspended in a solution having a pH above or below pH 7.0 for oral  
administration. The microparticles will remain intact and undissolved until they  
reached the intestines where the intestinal pH would cause them to dissolve thereby  
releasing the antitoxin.



The invention contemplates a method of treatment which can be administered for treatment of acute intoxication. In one embodiment, antitoxin is administered orally in either a delivery solution or in tablet form, in therapeutic dosage, to a subject intoxicated by the bacterial toxin which served as immunogen for the antitoxin.

5           The invention also contemplates a method of treatment which can be administered prophylactically. In one embodiment, antitoxin is administered orally, in a delivery solution, in therapeutic dosage, to a subject, to prevent intoxication of the subject by the bacterial toxin which served as immunogen for the production of antitoxin. In another embodiment, antitoxin is administered orally in solid form such  
10 as tablets or as microencapsulated particles. Microencapsulation of lyophilized antibody using compounds such as Eudragit® (Rohm GmbH) or polyethylene glycol , which dissolve at a wide range of pH units, allows the oral administration of solid antitoxin in a liquid form (*i.e.*, a suspension) to recipients unable to tolerate administration of tablets (*e.g.*, children or patients on feeding tubes). In one preferred  
15 embodiment the subject is a child. In another embodiment, antibody raised against whole bacterial organism is administered orally to a subject, in a delivery solution, in therapeutic dosage.

#### **V.     Vaccines Against Clostridial Species**

20           The invention contemplates the generation of mono- and multivalent vaccines for the protection of an animal (particularly humans) against several clostridial species. Of particular interest are vaccines which stimulate the production of a humoral immune response to *C. botulinum*, *C. tetani* and *C. difficile* in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the clostridial species listed above. When toxin proteins are used as  
25 immunogens they are generally modified to reduce the toxicity. This modification may be by chemical or genetic (*i.e.*, recombinant DNA technology) means. In general genetic detoxification (*i.e.*, the expression of nontoxic fragments in a host cell) is preferred as the expression of nontoxic fragments in a host cell precludes the presence

of intact, active toxin in the final preparation. However, when chemical modification is desired, the preferred toxin modification is formaldehyde treatment.

5 The invention contemplates that recombinant *C. botulinum* toxin proteins be used as antigens in mono- and multivalent vaccine preparations. Soluble, substantially endotoxin-free recombinant *C. botulinum* toxin proteins derived from serotypes A, B and E may be used individually (*i.e.*, as mono-valent vaccines) or in combination (*i.e.*, as a multi-valent vaccine). In addition, the recombinant *C. botulinum* toxin proteins derived from serotypes A, B and E may be used in conjunction with either recombinant or native toxins or toxoids from other serotypes of *C. botulinum*, *C. difficile* and *C.*  
10 *tetani* as antigens for the preparation of these mono- and multivalent vaccines. It is contemplated that, due to the structural similarity of *C. botulinum* and *C. tetani* toxin proteins, a vaccine comprising *C. difficile* and *botulinum* toxin proteins (native or recombinant or a mixture thereof) be used to stimulate an immune response against *C. botulinum*, *C. tetani* and *C. difficile*.

15 The present invention further contemplates multi-valent vaccines comprising two or more botulinal toxin proteins selected from the group comprising recombinant *C. botulinum* toxin proteins derived from serotypes A, B, C (including C1 and C2), D, E, F and G.

20 The adverse consequences of exposure to botulinal toxin would be avoided by immunization of subjects at risk of exposure to the toxin with nontoxic preparations which confer immunity such as chemically or genetically detoxified toxin.

Vaccines which confer immunity against one or more of the toxin types A, B, E, F and G would be useful as a means of protecting humans from the deleterious effects of those *C. botulinum* toxins known to affect man. Indeed as the possibility  
25 exists that humans could be exposed to any of the seven serotypes of *C. botulinum* toxin (*e.g.*, during biological warfare or the production of toxin in a laboratory setting), multivalent vaccines capable of conferring immunity against toxin types A-G (including both C1 and C2 toxins) would be useful for the protection of humans.

Vaccines which confer immunity against one or more of the toxin types C, D and E would be useful for veterinary applications.

5 The botulinal neurotoxin is synthesized as a single polypeptide chain which is processed into a heavy (H; ~100 kD) and a light (L; ~50 kD) chain by cleavage with proteolytic enzymes; these two chains are held together via disulfide bonds in the active toxin (referred to as derivative toxin) [B.R. DasGupta and H. Sugiyama, Biochem. Biophys. Res. Commun. 48:108 (1972); reviewed in B.R. DasGupta, J. Physiol. 84:220 (1990), H. Sugiyama, Microbiol. Rev. 44:419 (1980) and C.L. Hatheway, Clin. Microbiol. Rev. 3:66 (1990)]. The heavy chain of the active toxin is  
10 cleaved by trypsin to produce two fragments termed H<sub>C</sub> (also referred to as H<sub>1</sub> or C) and H<sub>N</sub> (also referred to as H<sub>2</sub> or B). The H<sub>C</sub> fragment (~46 kD) comprises the carboxy end of the H chain. The H<sub>N</sub> fragment (~49 kD) comprises the amino end and remains attached to the L chain (H<sub>N</sub>L). Neither H<sub>C</sub> or H<sub>N</sub>L is toxic. H<sub>C</sub> competes with whole derivative toxin for binding to synaptosomes and therefore H<sub>C</sub> is said to contain the  
15 receptor binding site. The H<sub>C</sub> and H<sub>N</sub> fragments of botulinal toxin are analogous to the fragments C and B of tetanus toxin which are produced by papain cleavage. The C fragment of tetanus toxin has been shown to be responsible for the binding of tetanus toxin to purified gangliosides and neuronal cells [Halpern and Loftus, J. Biol. Chem. 288:11188 (1993)].

20 Antisera raised against purified preparations of isolated botulinal H and L chains have been shown to protect mice against the lethal effects of the toxin; however, the effectiveness of the two antisera differ with the anti-H sera being more potent (H. Sugiyama, *supra*). While the different botulinal toxins show structural similarity to one another, the different serotypes are reported to be immunologically  
25 distinct (*i.e.*, sera raised against one toxin type does not cross-react to a significant degree with other types). Thus, the generation of multivalent vaccines may require the use of more than one type of toxin.

*C. botulinum* toxin genes from all seven serotypes have been cloned and sequenced (Minton (1995), *supra*); in addition, partial amino acid sequence is available

for a number of *C. botulinum* toxins isolated from different strains within a given serotype. The *C. botulinum* toxins contain about 1250-1300 amino acid residues. On the DNA level, the overall degree of homology between *C. botulinum* serotypes A, B, C, D and E toxins averages between 50 and 60% identity with a greater degree of homology being found between H chain-encoding regions than between those encoding L chains [Whelan *et al.* (1992) Appl. Environ. Microbiol. 58:2345]. The degree of identity between *C. botulinum* toxins on the amino acid level reflects the level of DNA sequence homology. The most divergent area of DNA and amino acid sequence is found within the carboxy-terminal area of the various *C. botulinum* H chain genes. This portion of the toxin (*i.e.*, H<sub>C</sub> or the C fragment) plays a major role in cell binding. As toxin from different serotypes is thought to bind to distinct cell receptor molecules, it is not surprising that the toxins diverge significantly over this region.

Within a given serotype, small variations in the primary amino acid sequence of the botulinal toxins isolated from different strains has been reported [Whelan *et al.* (1992), *supra* and Minton (1995), *supra*]. The present invention contemplates fusion proteins comprising portions of *C. botulinum* toxins from serotypes A-G including the variants found among different strains within a given serotype. The present invention provides oligonucleotide primers which may be used to amplify the C fragment or receptor-binding region of the toxin gene from various strains of *C. botulinum* serotype A, serotype B, serotype C (C1 and C2), serotype D, serotype E, serotype F and serotype G. A large number of different strains of *C. botulinum* serotype A, serotype B, serotype C, serotype D serotype E and serotype F are available from the American Type Culture Collection (ATCC; Rockville, MD). For example, the ATCC provides the following: Type A strains: 174 (ATCC 3502), 457 (ATCC 17862), and NCTC 7272 (ATCC 19397); Type B strains: 34 (ATCC 439), 62A (ATCC 7948), NCA 213 B (ATCC 7949), 13114 (ATCC 8083), 3137 (ATCC 17780), 1347 (ATCC 17841), 2017 (ATCC 17843), 2217 (ATCC 17844), 2254 (ATCC 17845) and VP 1731 (ATCC 25765); Type C strains: 2220 (ATCC 17782), 2239 (ATCC 17783), 2223 (ATCC 17784; a type C- $\beta$  strain; C- $\beta$  strains produce C2 toxin), 662 (ATCC 17849; a type C-

$\alpha$  strain; C- $\alpha$  strains produce mainly C1 toxin and a small amount of C2 toxin), 2021 (ATCC 17850; a type C- $\alpha$  strain) and VPI 3803 (ATCC 25766); Type D strains: ATCC 9633, 2023 (ATCC 17851), and VPI 5995 (ATCC 27517); Type E strains: ATCC 43181, 36208 (ATCC 9564), 2231 (ATCC 17786), 2229 (ATCC 17852), 2279 (ATCC 17854) and 2285 (ATCC 17855) and Type F strains: 202F (ATCC 23387), VPI 4404 (ATCC 25764), VPI 2382 (ATCC 27321) and Langeland (ATCC 35415). Type G strain, 113/30 (NCFB 3012) may be obtained from the National Collection of Food Bacteria (NCFB, AFRC Institute of Food Research, Reading, United Kingdom).

Purification methods have been reported for native toxin types A, B, C, D, E, and F [reviewed in G. Sakaguchi, *Pharmac. Ther.* 19:165 (1983)]. As the different botulinal toxins are structurally related, the invention contemplates the expression of any of the botulinal toxins (*e.g.*, types A-G) as soluble recombinant fusion proteins.

In particular, methods for purification of the type A botulinum neurotoxin have been developed [L.J. Moberg and H. Sugiyama, *Appl. Environ. Microbiol.* 35:878 (1978)]. Immunization of hens with detoxified purified protein results in the generation of neutralizing antibodies [B.S. Thalley *et al.*, in *Botulinum and Tetanus Neurotoxins*, B.R. DasGupta, ed., Plenum Press, New York (1993), p. 467].

The currently available *C. botulinum* pentavalent vaccine comprising chemically inactivated (*i.e.*, formaldehyde treated) type A, B, C, D and E toxins is not adequate. The efficacy is variable (in particular, only 78% of recipients produce protective levels of anti-type B antibodies following administration of the primary series) and immunization is painful (deep subcutaneous inoculation is required for administration), with adverse reactions being common (moderate to severe local reactions occur in approximately 6% of recipients upon initial injection; this number rises to approximately 11% of individuals who receive booster injections) [Informational Brochure for the Pentavalent (ABCDE) Botulinum Toxoid, Centers for Disease Control]. Preparation of this vaccine is dangerous as active toxin must be handled by laboratory workers.

In general, chemical detoxification of bacterial toxins using agents such as formaldehyde, glutaraldehyde or hydrogen peroxide is not optimal for the generation of vaccines or antitoxins. A delicate balance must be struck between too much and too little chemical modification. If the treatment is insufficient, the vaccine may retain residual toxicity. If the treatment is too excessive, the vaccine may lose potency due to destruction of native immunogenic determinants. Another major limitation of using botulinal toxoids for the generation of antitoxins or vaccines is the high production expense. For the above reasons, the development of methods for the production of nontoxic but immunogenic *C. botulinum* toxin proteins is desirable.

The *C. botulinum* and *C. tetanus* toxin proteins have similar structures [reviewed in E.J. Schantz and E.A. Johnson, Microbiol. Rev. 56:80 (1992)]. The carboxy-terminal 50 kD fragment of the tetanus toxin heavy chain (fragment C) is released by papain cleavage and has been shown to be non-toxic and immunogenic. Recombinant tetanus toxin fragment C has been developed as a candidate vaccine antigen [A.J. Makoff *et al.*, Bio/Technology 7:1043 (1989)]. Mice immunized with recombinant tetanus toxin fragment C were protected from challenge with lethal doses of tetanus toxin. No studies have demonstrated that the recombinant tetanus fragment C protein confers immunity against other botulinal toxins such as the *C. botulinum* toxins.

Recombinant tetanus fragment C has been expressed in *E. coli* (A.J. Makoff *et al.*, Bio/Technology, *supra* and Nucleic Acids Res. 17:10191 (1989); J.L. Halpern *et al.*, Infect. Immun. 58:1004 (1990)), yeast [M.A. Romanos *et al.*, Nucleic Acids Res. 19:1461 (1991)] and baculovirus [I.G. Charles *et al.*, Infect. Immun. 59:1627 (1991)]. Synthetic tetanus toxin genes had to be constructed to facilitate expression in yeast (M.A. Romanos *et al.*, *supra*) and *E. coli* [A.J. Makoff *et al.*, Nucleic Acids Res., *supra*], due to the high A-T content of the tetanus toxin gene sequences. High A-T content is a common feature of clostridial genes (M.R. Popoff *et al.*, Infect. Immun. 59:3673 (1991); H.F. LaPenotiere *et al.*, in *Botulinum and Tetanus Neurotoxins*, B.R. DasGupta, ed., Plenum Press, New York (1993), p. 463] which creates expression

difficulties in *E. coli* and yeast due primarily to altered codon usage frequency and fortuitous polyadenylation sites, respectively.

The C fragment of the *C. botulinum* type A neurotoxin heavy chain has been evaluated as a vaccine candidate. The *C. botulinum* type A neurotoxin gene has been  
5 cloned and sequenced [D.E. Thompson *et al.*, Eur. J. Biochem. 189:73 (1990)]. The C fragment of the type A toxin was expressed as either a fusion protein comprising the botulinal C fragment fused with the maltose binding protein (MBP) or as a native protein [H.F. LaPenotiere *et al.*, (1993) *supra*, H.F. LaPenotiere *et al.*, Toxicon. 33:1383 (1995) and Middlebrook and Brown (1995), Curr. Top. Microbiol. Immunol.  
10 195:89-122]. The plasmid construct encoding the native protein was reported to be unstable, while the fusion protein was expressed primarily in inclusion bodies as insoluble protein. Immunization of mice with crudely purified MBP fusion protein resulted in protection against IP challenge with 3 LD<sub>50</sub> doses of toxin [LaPenotiere *et al.*, (1993) and (1995), *supra*]. However, this recombinant *C. botulinum* type A toxin  
15 C fragment/MBP fusion protein is not a suitable immunogen for the production of vaccines as it is expressed as an insoluble protein in *E. coli*. Furthermore, this recombinant *C. botulinum* type A toxin C fragment/MBP fusion protein was not shown to be substantially free of endotoxin contamination. Experience with recombinant *C. botulinum* type A toxin C fragment/MBP fusion proteins shows that the presence of  
20 the MBP on the fusion protein greatly complicates the removal of endotoxin from preparations of the recombinant fusion protein (*see* Ex. 24, *infra*). Expression of a synthetic gene encoding *C. botulinum* type A toxin C fragment as a soluble protein excreted from insect cells has been reported [Middlebrook and Brown (1995), *supra*]; no details regarding the level of expression achieved or the presence of endotoxin or  
25 other pyrogens were provided. Like the insoluble protein expressed in *E. coli*, immunization with the recombinant protein produced in insect cells was reported to protect mice from challenge with *C. botulinum* toxin A.

Inclusion body protein must be solubilized prior to purification and/or administration to a host. The harsh treatment of inclusion body protein needed to

accomplish this solubilization may reduce the immunogenicity of the purified protein. Ideally, recombinant proteins to be used as vaccines are expressed as soluble proteins at high levels (*i.e.*, greater than or equal to about 0.75% of total cellular protein) in *E. coli* or other host cells (*e.g.*, yeast, insect cells, etc.). This facilitates the production and isolation of sufficient quantities of the immunogen in a highly purified form (*i.e.*, substantially free of endotoxin or other pyrogen contamination). The ability to express recombinant toxin proteins as soluble proteins in *E. coli* is advantageous due to the low cost of growth compared to insect or mammalian tissue culture cells.

The *C. botulinum* type B neurotoxin gene has been cloned and sequenced from two strains of *C. botulinum* type B [Whelan *et al.* (1992) Appl. Environ. Microbiol. 58:2345 (Danish strain) and Hutson *et al.* (1994) Curr. Microbiol. 28:101 (Eklund 17B strain)]. The nucleotide sequence of the toxin gene derived from the Eklund 17B strain (ATCC 25765) is available from the EMBL/GenBank sequence data banks under the accession number X71343; the nucleotide sequence of the coding region is listed in SEQ ID NO:39. The amino acid sequence of the *C. botulinum* type B neurotoxin derived from the strain Eklund 17B is listed in SEQ ID NO:40. The nucleotide sequence of the *C. botulinum* serotype B toxin gene derived from the Danish strain is listed in SEQ ID NO:41. The amino acid sequence of the *C. botulinum* type B neurotoxin derived from the Danish strain is listed in SEQ ID NO:42.

The *C. botulinum* type B neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds. The light chain is responsible for pharmacological activity (*i.e.*, inhibition of the release of acetylcholine at the neuromuscular junction). The N-terminal portion of the heavy chain is thought to mediate channel formation while the C-terminal portion mediates toxin binding; the type B neurotoxin has been reported to exist as a mixture of predominantly single chain with some double chain (Whelan *et al.*, *supra*). The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H<sub>C</sub> domain. The present invention reports for the first time, the



expression of the C fragment of *C. botulinum* type B toxin in heterologous hosts (*e.g.*, *E. coli*).

5 The *C. botulinum* type E neurotoxin gene has been cloned and sequenced from a number of different strains [Poulet *et al.* (1992) Biochem. Biophys. Res. Commun. 183:107; Whelan *et al.* (1992) Eur. J. Biochem. 204:657; and Fujii *et al.* (1993) J. Gen. Microbiol. 139:79]. The nucleotide sequence of the type E toxin gene is available from the EMBL sequence data bank under accession numbers X62089 (strain Beluga) and X62683 (strain NCTC 11219); the nucleotide sequence of the coding region (strain Beluga) is listed in SEQ ID NO:45. The amino acid sequence of the *C. botulinum* type E neurotoxin derived from strain Beluga is listed in SEQ ID NO:46. The type E neurotoxin gene is synthesized as a single polypeptide chain which may be converted to a double-chain form (*i.e.*, a heavy chain and a light chain) by cleavage with trypsin; unlike the type A neurotoxin, the type E neurotoxin exists essentially only in the single-chain form. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H<sub>C</sub> domain. The present invention reports for the first time, the expression of the C fragment of *C. botulinum* type E toxin in heterologous hosts (*e.g.*, *E. coli*).

15 The *C. botulinum* type C1, D, F and G neurotoxin genes have been cloned and sequenced. The nucleotide and amino acid sequences of these genes and toxins are provided herein. The invention provides methods for the expression of the C fragment from each of these toxin genes in heterologous hosts and the purification of the resulting recombinant proteins.

20 The subject invention provides methods which allow the production of soluble *C. botulinum* toxin proteins in economical host cells (*e.g.*, *E. coli*). In addition the subject invention provides methods which allow the production of soluble botulinal toxin proteins in yeast and insect cells. Further, methods for the isolation of purified soluble *C. botulinum* toxin proteins which are suitable for immunization of humans and other animals are provided. These soluble, purified preparations of *C. botulinum*

toxin proteins provide the basis for improved vaccine preparations and facilitate the production of antitoxin.

When recombinant clostridial toxin proteins produced in gram-negative bacteria (e.g., *E. coli*) are used as vaccines, they are purified to remove endotoxin prior to administration to a host animal. In order to vaccinate a host, an immunogenically-effective amount of purified substantially endotoxin-free recombinant clostridial toxin protein is administered in any of a number of physiologically acceptable carriers known to the art. When administered for the purpose of vaccination, the purified substantially endotoxin-free recombinant clostridial toxin protein may be used alone or in conjunction with known adjuvants, including potassium alum, aluminum phosphate, aluminum hydroxide, Gerbu adjuvant (GmDP; C.C. Biotech Corp.), RIBI adjuvant (MPL; RIBI Immunochemical Research, Inc.), QS21 (Cambridge Biotech). The alum and aluminum-based adjuvants are particularly preferred when vaccines are to be administered to humans; however, any adjuvant approved for use in humans may be employed. The route of immunization may be nasal, oral, intramuscular, intraperitoneal or subcutaneous.

The invention contemplates the use of soluble, substantially endotoxin-free preparations of fusion proteins comprising the C fragment of the *C. botulinum* type A, B, C, D, E, F, and G toxin as vaccines. In one embodiment, the vaccine comprises the C fragment of either the *C. botulinum* type A, B, C, D, E, F, or G toxin and a poly-histidine tract (also called a histidine tag). In a particularly preferred embodiment, a fusion protein comprising the histidine tagged C fragment is expressed using the pET series of expression vectors (Novagen). The pET expression system utilizes a vector containing the T7 promoter which encodes the fusion protein and a host cell which can be induced to express the T7 DNA polymerase (i.e., a DE3 host strain). The production of C fragment fusion proteins containing a histidine tract is not limited to the use of a particular expression vector and host strain. Several commercially available expression vectors and host strains can be used to express the C fragment protein sequences as a fusion protein containing a histidine tract (For example, the pQE series (pQE-8, 12, 16, 17, 18, 30, 31, 32, 40, 41, 42, 50, 51, 52, 60 and 70) of

expression vectors (Qiagen) which are used with the host strains M15[pREP4] (Qiagen) and SG13009[pREP4] (Qiagen) can be used to express fusion proteins containing six histidine residues at the amino-terminus of the fusion protein).

5 Furthermore a number of commercially available expression vectors which provide a histidine tract also provide a protease cleavage site between the histidine tract and the protein of interest (*e.g.*, botulinal toxin sequences). Cleavage of the resulting fusion protein with the appropriate protease will remove the histidine tag from the protein of interest (*e.g.*, botulinal toxin sequences) (see Example 28a, *infra*). Removal of the histidine tag may be desirable prior to administration of the recombinant botulinal toxin protein to a subject (*e.g.*, a human).

10

## VI. Detection Of Toxin

The invention contemplates detecting bacterial toxin in a sample. The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture. On the other hand, it is meant to include both biological and environmental samples.

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Biological samples may be animal, including human, fluid, solid (*e.g.*, stool) or tissue; liquid and solid food products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

20

The invention contemplates detecting bacterial toxin by a competitive immunoassay method that utilizes recombinant toxin A and toxin B proteins, antibodies raised against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (*e.g.*, a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated

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antibodies directed against the recombinant toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (*e.g.*, microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

It is also contemplated that bacterial toxin be detected by pouring liquids (*e.g.*, soups and other fluid foods and feeds including nutritional supplements for humans and other animals) over immobilized antibody which is directed against the bacterial toxin. It is contemplated that the immobilized antibody will be present in or on such supports as cartridges, columns, beads, or any other solid support medium. In one embodiment, following the exposure of the liquid to the immobilized antibody, unbound toxin is substantially removed by washing. The exposure of the liquid is then exposed to a reporter substance which detects the presence of bound toxin. In a preferred embodiment the reporter substance is an enzyme, fluorescent dye, or radioactive compound attached to an antibody which is directed against the toxin (*i.e.*,

in a "sandwich" immunoassay). It is also contemplated that the detection system will be developed as necessary (*e.g.*, the addition of enzyme substrate in enzyme systems; observation using fluorescent light for fluorescent dye systems; and quantitation of radioactivity for radioactive systems).

## 5      **EXPERIMENTAL**

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

10      In the disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); BBS-Tween (borate buffered saline containing Tween); BSA (bovine serum albumin); ELISA (enzyme-linked immunosorbent assay); CFA (complete Freund's adjuvant); IFA (incomplete Freund's adjuvant); IgG (immunoglobulin G); IgY (immunoglobulin Y); IM (intramuscular); IP (intraperitoneal); IV (intravenous or intravascular); SC (subcutaneous); H<sub>2</sub>O (water);  
15      HCl (hydrochloric acid); LD<sub>100</sub> (lethal dose for 100% of experimental animals); aa (amino acid); HPLC (high performance liquid chromatography); kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); MgCl<sub>2</sub> (magnesium chloride); NaCl (sodium chloride); Na<sub>2</sub>CO<sub>3</sub> (sodium carbonate); OD<sub>280</sub> (optical density at 280 nm); OD<sub>600</sub> (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS [phosphate buffered saline (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2)]; PEG (polyethylene glycol); PMSF (phenylmethylsulfonyl fluoride); SDS (sodium dodecyl sulfate); Tris  
20      (tris(hydroxymethyl)aminomethane); Ensure® (Ensure®, Ross Laboratories, Columbus OH); Enfamil® (Enfamil®, Mead Johnson); w/v (weight to volume); v/v (volume to volume); Amicon (Amicon, Inc., Beverly, MA); Amresco (Amresco, Inc., Solon, OH); ATCC (American Type Culture Collection, Rockville, MD); BBL (Baltimore Biologics Laboratory, (a division of Becton Dickinson), Cockeysville, MD); Becton Dickinson  
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(Becton Dickinson Labware, Lincoln Park, NJ); BioRad (BioRad, Richmond, CA);  
Biotech (C-C Biotech Corp., Poway, CA); Charles River (Charles River Laboratories,  
Wilmington, MA); Cocalico (Cocalico Biologicals Inc., Reamstown, PA); CytRx  
(CytRx Corp., Norcross, GA); Falcon (e.g. Baxter Healthcare Corp., McGaw Park, IL  
5 and Becton Dickinson); FDA (Federal Food and Drug Administration); Fisher Biotech  
(Fisher Biotech, Springfield, NJ); GIBCO (Grand Island Biologic Company/BRL,  
Grand Island, NY); Gibco-BRL (Life Technologies, Inc., Gaithersburg, MD); Harlan  
Sprague Dawley (Harlan Sprague Dawley, Inc., Madison, WI); Mallinckrodt (a  
division of Baxter Healthcare Corp., McGaw Park, IL); Millipore (Millipore Corp.,  
10 Marlborough, MA); New England Biolabs (New England Biolabs, Inc., Beverly, MA);  
Novagen (Novagen, Inc., Madison, WI); Pharmacia (Pharmacia, Inc., Piscataway, NJ);  
Qiagen (Qiagen, Chatsworth, CA); Sasco (Sasco, Omaha, NE); Showdex (Showa  
Denko America, Inc., New York, NY); Sigma (Sigma Chemical Co., St. Louis, MO);  
Sterogene (Sterogene, Inc., Arcadia, CA); Tech Lab (Tech Lab, Inc., Blacksburg,  
15 VA); and Vaxcell (Vaxcell, Inc., a subsidiary of CytRX Corp., Norcross, GA).

When a recombinant protein is described in the specification it is referred to in  
a short-hand manner by the amino acids in the toxin sequence present in the  
recombinant protein rounded to the nearest 10. For example, the recombinant protein  
pMB1850-2360 contains amino acids 1852 through 2362 of the *C. difficile* toxin B  
20 protein. The specification gives detailed construction details for all recombinant  
proteins such that one skilled in the art will know precisely which amino acids are  
present in a given recombinant protein.

### EXAMPLE 1

#### Production Of High-Titer Antibodies To *Clostridium difficile* Organisms In A Hen

25 Antibodies to certain pathogenic organisms have been shown to be effective in  
treating diseases caused by those organisms. It has not been shown whether antibodies  
can be raised, against *Clostridium difficile*, which would be effective in treating

infection by this organism. Accordingly, *C. difficile* was tested as immunogen for production of hen antibodies.

To determine the best course for raising high-titer egg antibodies against whole *C. difficile* organisms, different immunizing strains and different immunizing concentrations were examined. The example involved (a) preparation of the bacterial immunogen, (b) immunization, (c) purification of anti-bacterial chicken antibodies, and (d) detection of anti-bacterial antibodies in the purified IgY preparations.

#### a) Preparation Of Bacterial Immunogen

*C. difficile* strains 43594 (serogroup A) and 43596 (serogroup C) were originally obtained from the ATCC. These two strains were selected because they represent two of the most commonly-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee *et al.*, J. Clin. Microbiol., 28(10):2210 (1990).] Additionally, both of these strains have been previously characterized with respect to their virulence in the Syrian hamster model for *C. difficile* infection. [Delmee *et al.*, J. Med Microbiol., 33:85 (1990).]

The bacterial strains were separately cultured on brain heart infusion agar for 48 hours at 37°C in a Gas Pack 100 Jar (BBL, Cockeysville, MD) equipped with a Gas Pack Plus anaerobic envelope (BBL). Forty-eight hour cultures were used because they produce better growth and the organisms have been found to be more cross-reactive with respect to their surface antigen presentation. The greater the degree of cross-reactivity of our IgY preparations, the better the probability of a broad range of activity against different strains/serogroups. [Toma *et al.*, J. Clin. Microbiol., 26(3):426 (1988).]

The resulting organisms were removed from the agar surface using a sterile dacron-tip swab, and were suspended in a solution containing 0.4% formaldehyde in PBS, pH 7.2. This concentration of formaldehyde has been reported as producing good results for the purpose of preparing whole-organism immunogen suspensions for the generation of polyclonal anti-*C. difficile* antisera in rabbits. [Delmee *et al.*, J. Clin.

Microbiol., 21:323 (1985); Davies *et al.*, Microbial Path., 9:141 (1990).] In this manner, two separate bacterial suspensions were prepared, one for each strain. The two suspensions were then incubated at 4°C for 1 hour. Following this period of formalin-treatment, the suspensions were centrifuged at  $4,200 \times g$  for 20 min., and the resulting pellets were washed twice in normal saline. The washed pellets, which contained formalin-treated whole organisms, were resuspended in fresh normal saline such that the visual turbidity of each suspension corresponded to a #7 McFarland standard. [M.A.C. Edelstein, "Processing Clinical Specimens for Anaerobic Bacteria: Isolation and Identification Procedures," in S.M. Finegold *et al* (eds.), *Bailey and Scott's Diagnostic Microbiology*, pp. 477-507, C.V. Mosby Co., (1990). The preparation of McFarland nephelometer standards and the corresponding approximate number of organisms for each tube are described in detail at pp. 172-173 of this volume.] Each of the two #7 suspensions was then split into two separate volumes. One volume of each suspension was volumetrically adjusted, by the addition of saline, to correspond to the visual turbidity of a #1 McFarland standard. [*Id.*] The #1 suspensions contained approximately  $3 \times 10^8$  organisms/ml, and the #7 suspensions contained approximately  $2 \times 10^9$  organisms/ml. [*Id.*] The four resulting concentration-adjusted suspensions of formalin-treated *C. difficile* organisms were considered to be "bacterial immunogen suspensions." These suspensions were used immediately after preparation for the initial immunization. [*See* section (b).]

The formalin-treatment procedure did not result in 100% non-viable bacteria in the immunogen suspensions. In order to increase the level of killing, the formalin concentration and length of treatment were both increased for subsequent immunogen preparations, as described below in Table 3. (Although viability was decreased with the stronger formalin treatment, 100% inviability of the bacterial immunogen suspensions was not reached.) Also, in subsequent immunogen preparations, the formalin solutions were prepared in normal saline instead of PBS. At day 49, the day of the fifth immunization, the excess volumes of the four previous bacterial



immunogen suspensions were stored frozen at -70°C for use during all subsequent immunizations.

**b) Immunization**

For the initial immunization, 1.0 ml volumes of each of the four bacterial immunogen suspensions described above were separately emulsified in 1.2 ml volumes of CFA (GIBCO). For each of the four emulsified immunogen suspensions, two four-month old White Leghorn hens (pre-laying) were immunized. (It is not necessary to use pre-laying hens; actively-laying hens can also be utilized.) Each hen received a total volume of approximately 1.0 ml of a single emulsified immunogen suspension via four injections (two subcutaneous and two intramuscular) of approximately 250 µl per site. In this manner, a total of four different immunization combinations, using two hens per combination, were initiated for the purpose of evaluating both the effect of immunizing concentration on egg yolk antibody (IgY) production, and interstrain cross-reactivity of IgY raised against heterologous strains. The four immunization groups are summarized in Table 3.

**TABLE 3**

Immunization Groups

Group Designation	Immunizing Strain	Approximate Immunizing Dose
CD 43594, #1	<i>C. difficile</i> strain 43594	$1.5 \times 10^8$ organisms/hen
CD 43594, #7	" "	$1.0 \times 10^9$ organisms/hen
CD 43596, #1	<i>C. difficile</i> strain 43596	$1.5 \times 10^8$ organisms/hen
CD 43596, #7	" "	$1.0 \times 10^9$ organisms/hen

The time point for the first series of immunizations was designated as "day zero." All subsequent immunizations were performed as described above except that the bacterial immunogen suspensions were emulsified using IFA (GIBCO) instead of CFA, and for the later time point immunization, the stored frozen suspensions were

used instead of freshly-prepared suspensions. The immunization schedule used is listed in Table 4.

**TABLE 4**

Immunization Schedule

Day Of Immunization	Formalin-Treatment	Immunogen Preparation Used
0	1%, 1 hr.	freshly-prepared
14	1%, overnight	" "
21	1%, overnight	" "
35	1%, 48 hrs.	" "
49	1%, 72 hrs.	" "
70	" "	stored frozen
85	" "	" "
105	" "	" "

**c) Purification Of Anti-Bacterial Chicken Antibodies**

Groups of four eggs were collected per immunization group between days 80 and 84 post-initial immunization, and chicken immunoglobulin (IgY) was extracted according to a modification of the procedure of A. Polson *et al.*, Immunol. Comm., 9:495 (1980). A gentle stream of distilled water from a squirt bottle was used to separate the yolks from the whites, and the yolks were broken by dropping them through a funnel into a graduated cylinder. The four individual yolks were pooled for each group. The pooled, broken yolks were blended with 4 volumes of egg extraction buffer to improve antibody yield (egg extraction buffer is 0.01 M sodium phosphate, 0.1 M NaCl, pH 7.5, containing 0.005% thimerosal), and PEG 8000 (Amresco) was added to a concentration of 3.5%. When all the PEG dissolved, the protein precipitates that formed were pelleted by centrifugation at  $13,000 \times g$  for 10 minutes. The supernatants were decanted and filtered through cheesecloth to remove the lipid layer, and the PEG was added to the supernatants to a final concentration of 12% (the supernatants were assumed to contain 3.5% PEG). After a second centrifugation, the supernatants were discarded and the pellets were centrifuged a final time to extrude the

remaining PEG. These crude IgY pellets were then dissolved in the original yolk volume of egg extraction buffer and stored at 4°C. As an additional control, a preimmune IgY solution was prepared as described above, using eggs collected from unimmunized hens.

5                    **d)      Detection Of Anti-Bacterial Antibodies In The  
Purified IgY Preparations**

In order to evaluate the relative levels of specific anti-*C. difficile* activity in the IgY preparations described above, a modified version of the whole-organism ELISA procedure of N.V. Padhye *et al.*, J. Clin. Microbiol. 29:99-103 (1990) was used.

10      Frozen organisms of both *C. difficile* strains described above were thawed and diluted to a concentration of approximately  $1 \times 10^7$  organisms/ml using PBS, pH 7.2. In this way, two separate coating suspensions were prepared, one for each immunizing strain. Into the wells of 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were placed 100 µl volumes of the coating suspensions. In this manner, each plate well received a  
15      total of approximately  $1 \times 10^6$  organisms of one strain or the other. The plates were then incubated at 4°C overnight. The next morning, the coating suspensions were decanted, and all wells were washed three times using PBS. In order to block non-specific binding sites, 100 µl of 0.5% BSA (Sigma) in PBS was then added to each well, and the plates were incubated for 2 hours at room temperature. The blocking  
20      solution was decanted, and 100 µl volumes of the IgY preparations described above were initially diluted 1:500 with a solution of 0.1% BSA in PBS, and then serially diluted in 1:5 steps. The following dilutions were placed in the wells: 1:500, 1:2,500, 1:62,500, 1:312,500, and 1:1,562,500. The plates were again incubated for 2 hours at room temperature. Following this incubation, the IgY-containing solutions were  
25      decanted, and the wells were washed three times using BBS-Tween (0.1 M boric acid, 0.025 M sodium borate, 1.0 M NaCl, 0.1% Tween-20), followed by two washes using PBS-Tween (0.1% Tween-20), and finally, two washes using PBS only. To each well, 100 µl of a 1:750 dilution of rabbit anti-chicken IgG (whole-molecule)-alkaline phosphatase conjugate (Sigma) (diluted in 0.1% BSA in PBS) was added. The plates

were again incubated for 2 hours at room temperature. The conjugate solutions were decanted and the plates were washed as described above, substituting 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5 for the PBS in the final wash. The plates were developed by the addition of 100 µl of a solution containing 1 mg/ml para-nitrophenyl phosphate (Sigma) dissolved in 50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH 9.5 to each well, and incubating the plates at room temperature in the dark for 45 minutes. The absorbance of each well was measured at 410 nm using a Dynatech MR 700 plate reader. In this manner, each of the four IgY preparations described above was tested for reactivity against both of the immunizing *C. difficile* strains; strain-specific, as well as cross-reactive activity was determined.

Table 5 shows the results of the whole-organism ELISA. All four IgY preparations demonstrated significant levels of activity, to a dilution of 1:62,500 or greater against both of the immunizing organism strains. Therefore, antibodies raised against one strain were highly cross-reactive with the other strain, and vice versa. The immunizing concentration of organisms did not have a significant effect on organism-specific IgY production, as both concentrations produced approximately equivalent responses. Therefore, the lower immunizing concentration of approximately  $1.5 \times 10^8$  organisms/hen is the preferred immunizing concentration of the two tested. The preimmune IgY preparation appeared to possess relatively low levels of *C. difficile*-reactive activity to a dilution of 1:500, probably due to prior exposure of the animals to environmental clostridia.

An initial whole-organism ELISA was performed using IgY preparations made from single CD 43594, #1 and CD 43596, #1 eggs collected around day 50 (data not shown). Specific titers were found to be 5 to 10-fold lower than those reported in Table 5. These results demonstrate that it is possible to begin immunizing hens prior to the time that they begin to lay eggs, and to obtain high titer specific IgY from the first eggs that are laid. In other words, it is not necessary to wait for the hens to begin laying before the immunization schedule is started.

TABLE 5

Results Of The Anti-*C. difficile* Whole-Organism ELISA

IgY Preparation	Dilution Of IgY Prep	43594-Coated Wells	43596-Coated Wells
CD 43594, #1	1:500	1.746	1.801
	1:2,500	1.092	1.670
	1:12,500	0.202	0.812
	1:62,500	0.136	0.179
	1:312,500	0.012	0.080
	1:1,562,500	0.002	0.020
CD 43594, #7	1:500	1.780	1.771
	1:2,500	1.025	1.078
	1:12,500	0.188	0.382
	1:62,500	0.052	0.132
	1:312,500	0.022	0.043
	1:1,562,500	0.005	0.024
CD 43596, #1	1:500	1.526	1.790
	1:2,500	0.832	1.477
	1:12,500	0.247	0.452
	1:62,500	0.050	0.242
	1:312,500	0.010	0.067
	1:1,562,500	0.000	0.036
CD 43596, #7	1:500	1.702	1.505
	1:2,500	0.706	0.866
	1:12,500	0.250	0.282
	1:62,500	0.039	0.078
	1:312,500	0.002	0.017
	1:1,562,500	0.000	0.010
Preimmune IgY	1:500	0.142	0.309
	1:2,500	0.032	0.077
	1:12,500	0.006	0.024
	1:62,500	0.002	0.012
	1:312,500	0.004	0.010
	1:1,562,500	0.002	0.014

## EXAMPLE 2

Treatment Of *C. difficile* Infection With Anti-*C. difficile* Antibody

In order to determine whether the immune IgY antibodies raised against whole *C. difficile* organisms were capable of inhibiting the infection of hamsters by *C. difficile*, hamsters infected by these bacteria were utilized. [Lyerly *et al.*, Infect. Immun., 59:2215-2218 (1991).] This example involved: (a) determination of the

lethal dose of *C. difficile* organisms; and (b) treatment of infected animals with immune antibody or control antibody in nutritional solution.

a) **Determination Of The Lethal Dose Of *C. difficile* Organisms**

5           Determination of the lethal dose of *C. difficile* organisms was carried out according to the model described by D.M. Lyster *et al.*, Infect. Immun., 59:2215-2218 (1991). *C. difficile* strain ATCC 43596 (serogroup C, ATCC) was plated on BHI agar and grown anaerobically (BBL Gas Pak 100 system) at 37°C for 42 hours. Organisms were removed from the agar surface using a sterile dacron-tip swab and suspended in  
10       sterile 0.9% NaCl solution to a density of  $10^8$  organisms/ml.

          In order to determine the lethal dose of *C. difficile* in the presence of control antibody and nutritional formula, non-immune eggs were obtained from unimmunized hens and a 12% PEG preparation made as described in Example 1(c). This preparation was redissolved in one fourth the original yolk volume of vanilla flavor  
15       Ensure®.

          Starting on day one, groups of female Golden Syrian hamsters (Harlan Sprague Dawley), 8-9 weeks old and weighing approximately 100 gm, were orally administered 1 ml of the preimmune/Ensure® formula at time zero, 2 hours, 6 hours, and 10 hours. At 1 hour, animals were orally administered 3.0 mg clindamycin HCl (Sigma) in 1 ml  
20       of water. This drug predisposes hamsters to *C. difficile* infection by altering the normal intestinal flora. On day two, the animals were given 1 ml of the preimmune IgY/Ensure® formula at time zero, 2 hours, 6 hours, and 10 hours. At 1 hour on day two, different groups of animals were inoculated orally with saline (control), or  $10^2$ ,  $10^4$ ,  $10^6$ , or  $10^8$  *C. difficile* organisms in 1 ml of saline. From days 3-12, animals were  
25       given 1 ml of the preimmune IgY/Ensure® formula three times daily and observed for the onset of diarrhea and death. Each animal was housed in an individual cage and was offered food and water *ad libitum*.

Administration of  $10^6$  -  $10^8$  organisms resulted in death in 3-4 days while the lower doses of  $10^2$  -  $10^4$  organisms caused death in 5 days. Cecal swabs taken from dead animals indicated the presence of *C. difficile*. Given the effectiveness of the  $10^2$  dose, this number of organisms was chosen for the following experiment to see if hyperimmune anti-*C. difficile* antibody could block infection.

**b) Treatment Of Infected Animals With Immune Antibody Or Control Antibody In Nutritional Formula**

The experiment in (a) was repeated using three groups of seven hamsters each. Group A received no clindamycin or *C. difficile* and was the survival control. Group B received clindamycin,  $10^2$  *C. difficile* organisms and preimmune IgY on the same schedule as the animals in (a) above. Group C received clindamycin,  $10^2$  *C. difficile* organisms, and hyperimmune anti-*C. difficile* IgY on the same schedule as Group B. The anti-*C. difficile* IgY was prepared as described in Example 1 except that the 12% PEG preparation was dissolved in one fourth the original yolk volume of Ensure®.

All animals were observed for the onset of diarrhea or other disease symptoms and death. Each animal was housed in an individual cage and was offered food and water *ad libitum*. The results are shown in Table 6.

**TABLE 6**

The Effect Of Oral Feeding Of Hyperimmune IgY Antibody on *C. difficile* Infection

Animal Group		Time To Diarrhea <sup>a</sup>	Time To Death <sup>a</sup>
A	pre-immune IgY only	no diarrhea	no deaths
B	Clindamycin, <i>C. difficile</i> , preimmune IgY	30 hrs.	49 hrs.
C	Clindamycin, <i>C. difficile</i> , immune IgY	33 hrs.	56 hrs.

<sup>a</sup> Mean of seven animals.

Hamsters in the control group A did not develop diarrhea and remained healthy during the experimental period. Hamsters in groups B and C developed diarrheal disease. Anti-*C. difficile* IgY did not protect the animals from diarrhea or death, all

animals succumbed in the same time interval as the animals treated with preimmune IgY. Thus, while immunization with whole organisms apparently can improve sublethal symptoms with particular bacteria (see U.S. Patent No. 5,080,895 to H. Tokoro), such an approach does not prove to be productive to protect against the lethal effects of *C. difficile*.

### EXAMPLE 3

#### Production of *C. botulinum* Type A Antitoxin in Hens

In order to determine whether antibodies could be raised against the toxin produced by clostridial pathogens, which would be effective in treating clostridial diseases, antitoxin to *C. botulinum* type A toxin was produced. This example involves: (a) toxin modification; (b) immunization; (c) antitoxin collection; (d) antigenicity assessment; and (e) assay of antitoxin titer.

##### a) Toxin Modification

*C. botulinum* type A toxoid was obtained from B. R. DasGupta. From this, the active type A neurotoxin (M.W. approximately 150 kD) was purified to greater than 99% purity, according to published methods. [B.R. DasGupta & V. Sathyamoorthy, Toxicon, 22:415 (1984).] The neurotoxin was detoxified with formaldehyde according to published methods. [B.R. Singh & B.R. DasGupta, Toxicon, 27:403 (1989).]

##### b) Immunization

*C. botulinum* toxoid for immunization was dissolved in PBS (1 mg/ml) and was emulsified with an approximately equal volume of CFA (GIBCO) for initial immunization or IFA for booster immunization. On day zero, two white leghorn hens, obtained from local breeders, were each injected at multiple sites (intramuscular and subcutaneous) with 1 ml inactivated toxoid emulsified in 1 ml CFA. Subsequent booster immunizations were made according to the following schedule for day of



injection and toxoid amount: days 14 and 21 - 0.5 mg; day 171 - 0.75 mg; days 394, 401, 409 - 0.25 mg. One hen received an additional booster of 0.150 mg on day 544.

**c) Antitoxin Collection**

5 Total yolk immunoglobulin (IgY) was extracted as described in Example 1(c) and the IgY pellet was dissolved in the original yolk volume of PBS with thimerosal.

**d) Antigenicity Assessment**

Eggs were collected from day 409 through day 423 to assess whether the toxoid was sufficiently immunogenic to raise antibody. Eggs from the two hens were pooled and antibody was collected as described in the standard PEG protocol.

10 [Example 1(c).] Antigenicity of the botulinal toxin was assessed on Western blots. The 150 kD detoxified type A neurotoxin and unmodified, toxic, 300 kD botulinal type A complex (toxin used for intragastric route administration for animal gut neutralization experiments; see Example 6) were separated on a SDS-polyacrylamide reducing gel. The Western blot technique was performed according to the method of

15 Towbin. [H. Towbin *et al.*, Proc. Natl. Acad. Sci. USA, 76:4350 (1979).] Ten  $\mu$ g samples of *C. botulinum* complex and toxoid were dissolved in SDS reducing sample buffer (1% SDS, 0.5% 2-mercaptoethanol, 50 mM Tris, pH 6.8, 10% glycerol, 0.025% w/v bromphenol blue, 10%  $\beta$ -mercaptoethanol), heated at 95°C for 10 min and separated on a 1 mm thick 5% SDS-polyacrylamide gel. [K. Weber and M.

20 Osborn, "Proteins and Sodium Dodecyl Sulfate: Molecular Weight Determination on Polyacrylamide Gels and Related Procedures," in *The Proteins*, 3d Edition (H. Neurath & R.L. Hill, eds), pp. 179-223, (Academic Press, NY, 1975).] Part of the gel was cut off and the proteins were stained with Coomassie Blue. The proteins in the remainder of the gel were transferred to nitrocellulose using the Milliblot-SDE electro-

25 blotting system (Millipore) according to manufacturer's directions. The nitrocellulose was temporarily stained with 10% Ponceau S [S.B. Carroll and A. Laughon, "Production and Purification of Polyclonal Antibodies to the Foreign Segment of  $\beta$ -

*galactosidase Fusion Proteins," in DNA Cloning: A Practical Approach, Vol.III,* (D. Glover, ed.), pp. 89-111, IRL Press, Oxford, (1987)] to visualize the lanes, then destained by running a gentle stream of distilled water over the blot for several minutes. The nitrocellulose was immersed in PBS containing 3% BSA overnight at 4°C to block any remaining protein binding sites.

The blot was cut into strips and each strip was incubated with the appropriate primary antibody. The avian anti-*C. botulinum* antibodies [described in (c)] and pre-immune chicken antibody (as control) were diluted 1:125 in PBS containing 1 mg/ml BSA for 2 hours at room temperature. The blots were washed with two changes each of large volumes of PBS, BBS-Tween and PBS, successively (10 min/wash). Goat anti-chicken IgG alkaline phosphatase conjugated secondary antibody (Fisher Biotech) was diluted 1:500 in PBS containing 1 mg/ml BSA and incubated with the blot for 2 hours at room temperature. The blots were washed with two changes each of large volumes of PBS and BBS-Tween, followed by one change of PBS and 0.1 M Tris-HCl, pH 9.5. Blots were developed in freshly prepared alkaline phosphatase substrate buffer (100 µg/ml nitroblue tetrazolium (Sigma), 50 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 5 mM MgCl<sub>2</sub> in 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5).

The Western blots are shown in Figure 1. The anti-*C. botulinum* IgY reacted to the toxoid to give a broad immunoreactive band at about 145-150 kD on the reducing gel. This toxoid is refractive to disulfide cleavage by reducing agents due to formalin crosslinking. The immune IgY reacted with the active toxin complex, a 97 kD *C. botulinum* type A heavy chain and a 53 kD light chain. The preimmune IgY was unreactive to the *C. botulinum* complex or toxoid in the Western blot.

#### e) Antitoxin Antibody Titer

The IgY antibody titer to *C. botulinum* type A toxoid of eggs harvested between day 409 and 423 was evaluated by ELISA, prepared as follows. Ninety-six-well Falcon Pro-bind plates were coated overnight at 4°C with 100 µl/well toxoid [B.R. Singh & B.R. Das Gupta, Toxicon 27:403 (1989)] at 2.5 µg/ml in PBS, pH 7.5

containing 0.005% thimerosal. The following day the wells were blocked with PBS containing 1% BSA for 1 hour at 37°C. The IgY from immune or preimmune eggs was diluted in PBS containing 1% BSA and 0.05% Tween 20 and the plates were incubated for 1 hour at 37°C. The plates were washed three times with PBS containing 0.05% Tween 20 and three times with PBS alone. Alkaline phosphatase-conjugated goat-anti-chicken IgG (Fisher Biotech) was diluted 1:750 in PBS containing 1% BSA and 0.05% Tween 20, added to the plates, and incubated 1 hour at 37°C. The plates were washed as before, and p-nitrophenyl phosphate (Sigma) at 1 mg/ml in 0.05 M Na<sub>2</sub>CO<sub>3</sub>, pH 9.5, 10 mM MgCl<sub>2</sub> was added.

The results are shown in Figure 2. Chickens immunized with the toxoid generated high titers of antibody to the immunogen. Importantly, eggs from both immunized hens had significant anti-immunogen antibody titers as compared to preimmune control eggs. The anti-*C. botulinum* IgY possessed significant activity, to a dilution of 1:93,750 or greater.

#### EXAMPLE 4

##### Preparation Of Avian Egg Yolk Immunoglobulin In An Orally Administrable Form

In order to administer avian IgY antibodies orally to experimental mice, an effective delivery formula for the IgY had to be determined. The concern was that if the crude IgY was dissolved in PBS, the saline in PBS would dehydrate the mice, which might prove harmful over the duration of the study. Therefore, alternative methods of oral administration of IgY were tested. The example involved: (a) isolation of immune IgY; (b) solubilization of IgY in water or PBS, including subsequent dialysis of the IgY-PBS solution with water to eliminate or reduce the salts (salt and phosphate) in the buffer; and (c) comparison of the quantity and activity of recovered IgY by absorbance at 280 nm and PAGE, and enzyme-linked immunoassay (ELISA).

**a) Isolation Of Immune IgY**

In order to investigate the most effective delivery formula for IgY, we used IgY which was raised against *Crotalus durissus terrificus* venom. Three eggs were collected from hens immunized with the *C. durissus terrificus* venom and IgY was  
5 extracted from the yolks using the modified Polson procedure described by Thalley and Carroll [Bio/Technology, 8:934-938 (1990)] as described in Example 1(c).

The egg yolks were separated from the whites, pooled, and blended with four volumes of PBS. Powdered PEG 8000 was added to a concentration of 3.5%. The mixture was centrifuged at 10,000 rpm for 10 minutes to pellet the precipitated  
10 protein, and the supernatant was filtered through cheesecloth to remove the lipid layer. Powdered PEG 8000 was added to the supernatant to bring the final PEG concentration to 12% (assuming a PEG concentration of 3.5% in the supernatant). The 12% PEG/IgY mixture was divided into two equal volumes and centrifuged to pellet the IgY.

**b) Solubilization Of The IgY In Water Or PBS**

15 One pellet was resuspended in 1/2 the original yolk volume of PBS, and the other pellet was resuspended in 1/2 the original yolk volume of water. The pellets were then centrifuged to remove any particles or insoluble material. The IgY in PBS solution dissolved readily but the fraction resuspended in water remained cloudy.

20 In order to satisfy anticipated sterility requirements for orally administered antibodies, the antibody solution needs to be filter-sterilized (as an alternative to heat sterilization which would destroy the antibodies). The preparation of IgY resuspended in water was too cloudy to pass through either a 0.2 or 0.45  $\mu$ m membrane filter, so 10 ml of the PBS resuspended fraction was dialyzed overnight at room temperature  
25 against 250 ml of water. The following morning the dialysis chamber was emptied and refilled with 250 ml of fresh H<sub>2</sub>O for a second dialysis. Thereafter, the yields of soluble antibody were determined at OD<sub>280</sub> and are compared in Table 7.

TABLE 7

Dependence Of IgY Yield On Solvents

Fraction	Absorbance Of 1:10 Dilution At 280 nm	Percent Recovery
PBS dissolved	1.149	100%
H <sub>2</sub> O dissolved	0.706	61%
PBS dissolved/H <sub>2</sub> O dialyzed	0.885	77%

Resuspending the pellets in PBS followed by dialysis against water recovered more antibody than directly resuspending the pellets in water (77% versus 61%). Equivalent volumes of the IgY preparation in PBS or water were compared by PAGE, and these results were in accordance with the absorbance values (data not shown).

#### c) Activity Of IgY Prepared With Different Solvents

An ELISA was performed to compare the binding activity of the IgY extracted by each procedure described above. *C. durissus terrificus* (*C.d.t.*) venom at 2.5 µg/ml in PBS was used to coat each well of a 96-well microtiter plate. The remaining protein binding sites were blocked with PBS containing 5 mg/ml BSA. Primary antibody dilutions (in PBS containing 1 mg/ml BSA) were added in duplicate. After 2 hours of incubation at room temperature, the unbound primary antibodies were removed by washing the wells with PBS, BBS-Tween, and PBS. The species specific secondary antibody (goat anti-chicken immunoglobulin alkaline-phosphatase conjugate (Sigma) was diluted 1:750 in PBS containing 1 mg/ml BSA and added to each well of the microtiter plate. After 2 hours of incubation at room temperature, the unbound secondary antibody was removed by washing the plate as before, and freshly prepared alkaline phosphatase substrate (Sigma) at 1 mg/ml in 50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH 9.5 was added to each well. The color development was measured on a Dynatech MR 700 microplate reader using a 412 nm filter. The results are shown in Table 8.

The binding assay results parallel the recovery values in Table 7, with PBS-dissolved IgY showing slightly more activity than the PBS-dissolved/H<sub>2</sub>O dialyzed

antibody. The water-dissolved antibody had considerably less binding activity than the other preparations.

### EXAMPLE 5

#### Survival Of Antibody Activity After Passage Through The Gastrointestinal Tract

5 In order to determine the feasibility of oral administration of antibody, it was of interest to determine whether orally administered IgY survived passage through the gastrointestinal tract. The example involved: (a) oral administration of specific immune antibody mixed with a nutritional formula; and (b) assay of antibody activity extracted from feces.

10 **TABLE 8**

Antigen-Binding Activity Of IgY Prepared With Different Solvents

Dilution	Preimmune	PBS Dissolved	H <sub>2</sub> O Dissolved	PBS/H <sub>2</sub> O
1:500	0.005	1.748	1.577	1.742
1:2,500	0.004	0.644	0.349	0.606
15 1:12,500	0.001	0.144	0.054	0.090
1:62,500	0.001	0.025	0.007	0.016
1:312,500	0.010	0.000	0.000	0.002

#### a) Oral Administration Of Antibody

20 The IgY preparations used in this example are the same PBS-dissolved/H<sub>2</sub>O dialyzed antivenom materials obtained in Example 4 above, mixed with an equal volume of Enfamil®. Two mice were used in this experiment, each receiving a different diet as follows:

- 1) water and food as usual;
  - 2) immune IgY preparation dialyzed against water and mixed 1:1 with
- 25 Enfamil®. (The mice were given the corresponding mixture as their only source of food and water).

**b) Antibody Activity After Ingestion**

After both mice had ingested their respective fluids, each tube was refilled with approximately 10 ml of the appropriate fluid first thing in the morning. By mid-morning there was about 4 to 5 ml of liquid left in each tube. At this point stool samples were collected from each mouse, weighed, and dissolved in approximately 500  $\mu$ l PBS per 100 mg stool sample. One hundred and sixty mg of control stools (no antibody) and 99 mg of experimental stools (specific antibody) in 1.5 ml microfuge tubes were dissolved in 800 and 500  $\mu$ l PBS, respectively. The samples were heated at 37°C for 10 minutes and vortexed vigorously. The experimental stools were also broken up with a narrow spatula. Each sample was centrifuged for 5 minutes in a microfuge and the supernatants, presumably containing the antibody extracts, were collected. The pellets were saved at 2-8°C in case future extracts were needed. Because the supernatants were tinted, they were diluted five-fold in PBS containing 1 mg/ml BSA for the initial dilution in the enzyme immunoassay (ELISA). The primary extracts were then diluted five-fold serially from this initial dilution. The volume of primary extract added to each well was 190  $\mu$ l. The ELISA was performed exactly as described in Example 4.

**TABLE 9**

Specific Antibody Activity After Passage Through The Gastrointestinal Tract

Dilution	Preimmune IgY	Control Fecal Extract	EXP. Fecal Extract
1:5	<0	0.000	0.032
1:25	0.016	<0	0.016
1:125	<0	<0	0.009
1:625	<0	0.003	0.001
1:3125	<0	<0	0.000

There was some active antibody in the fecal extract from the mouse given the specific antibody in Enfamil® formula, but it was present at a very low level. Since the samples were assayed at an initial 1:5 dilution, the binding observed could have been higher with less dilute samples. Consequently, the mice were allowed to

continue ingesting either regular food and water or the specific IgY in Enfamil® formula, as appropriate, so the assay could be repeated. Another ELISA plate was coated overnight with 5 µg/ml of *C.d.t.* venom in PBS.

5 The following morning the ELISA plate was blocked with 5 mg/ml BSA, and the fecal samples were extracted as before, except that instead of heating the extracts at 37°C, the samples were kept on ice to limit proteolysis. The samples were assayed undiluted initially, and in 5X serial dilutions thereafter. Otherwise the assay was carried out as before.

TABLE 10

Specific Antibody Survives Passage Through The Gastrointestinal Tract

10

15

Dilution	Preimmune IgY	Control Extract	Exp. Extract
undiluted	0.003	<0	0.379
1:5	<0	<0	0.071
1:25	0.000	<0	0.027
1:125	0.003	<0	0.017
1:625	0.000	<0	0.008
1:3125	0.002	<0	0.002

20 The experiment confirmed the previous results, with the antibody activity markedly higher. The control fecal extract showed no anti-*C.d.t.* activity, even undiluted, while the fecal extract from the anti-*C.d.t.* IgY/Enfamil®-fed mouse showed considerable anti-*C.d.t.* activity. This experiment (and the previous experiment) clearly demonstrate that active IgY antibody survives passage through the mouse digestive tract, a finding with favorable implications for the success of IgY antibodies administered orally as a therapeutic or prophylactic.



## EXAMPLE 6

### *In Vivo* Neutralization Of Type *C. botulinum* Type A Neurotoxin By Avian Antitoxin Antibody

This example demonstrated the ability of PEG-purified antitoxin, collected as described in Example 3, to neutralize the lethal effect of *C. botulinum* neurotoxin type A in mice. To determine the oral lethal dose (LD<sub>100</sub>) of toxin A, groups of BALB/c mice were given different doses of toxin per unit body weight (average body weight of 24 grams). For oral administration, toxin A complex, which contains the neurotoxin associated with other non-toxin proteins was used. This complex is markedly more toxic than purified neurotoxin when given by the oral route. [I. Ohishi *et al.*, Infect. Immun., 16:106 (1977).] *C. botulinum* toxin type A complex, obtained from Eric Johnson (University Of Wisconsin, Madison) was 250 µg/ml in 50 mM sodium citrate, pH 5.5, specific toxicity  $3 \times 10^7$  mouse LD<sub>50</sub>/mg with parenteral administration. Approximately 40-50 ng/gm body weight was usually fatal within 48 hours in mice maintained on conventional food and water. When mice were given a diet *ad libitum* of only Enfamil® the concentration needed to produce lethality was approximately 2.5 times higher (125 ng/gm body weight). Botulinal toxin concentrations of approximately 200 ng/gm body weight were fatal in mice fed Enfamil® containing preimmune IgY (resuspended in Enfamil® at the original yolk volume).

The oral LD<sub>100</sub> of *C. botulinum* toxin was also determined in mice that received known amounts of a mixture of preimmune IgY-Ensure® delivered orally through feeding needles. Using a 22 gauge feeding needle, mice were given 250 µl each of a preimmune IgY-Ensure® mixture (preimmune IgY dissolved in 1/4 original yolk volume) 1 hour before and 1/2 hour and 5 hours after administering botulinal toxin. Toxin concentrations given orally ranged from approximately 12 to 312 ng/gm body weight (0.3 to 7.5 µg per mouse). Botulinal toxin complex concentration of approximately 40 ng/gm body weight (1 µg per mouse) was lethal in all mice in less than 36 hours.

Two groups of BALB/c mice, 10 per group, were each given orally a single dose of 1 µg each of botulinal toxin complex in 100 µl of 50 mM sodium citrate pH 5.5. The mice received 250 µl treatments of a mixture of either preimmune or immune IgY in Ensure® (1/4 original yolk volume) 1 hour before and 1/2 hour, 4 hours, and 8 hours after botulinal toxin administration. The mice received three treatments per day for two more days. The mice were observed for 96 hours. The survival and mortality are shown in Table 11.

TABLE 11

Neutralization Of Botulinal Toxin A *In Vivo*

Toxin Dose ng/gm	Antibody Type	Number Of Mice Alive	Number Of Mice Dead
41.6	non-immune	0	10
41.6	anti-botulinal toxin	10	0

All mice treated with the preimmune IgY-Ensure® mixture died within 46 hours post-toxin administration. The average time of death in the mice was 32 hours post toxin administration. Treatments of preimmune IgY-Ensure® mixture did not continue beyond 24 hours due to extensive paralysis of the mouth in mice of this group. In contrast, all ten mice treated with the immune anti-botulinal toxin IgY-Ensure® mixture survived past 96 hours. Only 4 mice in this group exhibited symptoms of botulism toxicity (two mice about 2 days after and two mice 4 days after toxin administration). These mice eventually died 5 and 6 days later. Six of the mice in this immune group displayed no adverse effects to the toxin and remained alive and healthy long term. Thus, the avian anti-botulinal toxin antibody demonstrated very good protection from the lethal effects of the toxin in the experimental mice.

## EXAMPLE 7

### Production Of An Avian Antitoxin Against *Clostridium difficile* Toxin A

Toxin A is a potent cytotoxin secreted by pathogenic strains of *C. difficile*, that plays a direct role in damaging gastrointestinal tissues. In more severe cases of *C. difficile* intoxication, pseudomembranous colitis can develop which may be fatal. This would be prevented by neutralizing the effects of this toxin in the gastrointestinal tract. As a first step, antibodies were produced against a portion of the toxin. The example involved: (a) conjugation of a synthetic peptide of toxin A to bovine serum albumin; (b) immunization of hens with the peptide-BSA conjugate; and (c) detection of antitoxin peptide antibodies by ELISA.

#### a) Conjugation Of A Synthetic Peptide Of Toxin A To Bovine Serum Albumin

The synthetic peptide CQTIDGKKYYFN-NH<sub>2</sub> (SEQ ID NO:82) was prepared commercially (Multiple Peptide Systems, San Diego, CA) and validated to be >80% pure by high-pressure liquid chromatography. The eleven amino acids following the cysteine residue represent a consensus sequence of a repeated amino acid sequence found in Toxin A. [Wren *et al.*, Infect. Immun., 59:3151-3155 (1991).] The cysteine was added to facilitate conjugation to carrier protein.

In order to prepare the carrier for conjugation, BSA (Sigma) was dissolved in 0.01 M NaPO<sub>4</sub>, pH 7.0 to a final concentration of 20 mg/ml and n-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Pierce) was dissolved in N,N-dimethyl formamide to a concentration of 5 mg/ml. MBS solution, 0.51 ml, was added to 3.25 ml of the BSA solution and incubated for 30 minutes at room temperature with stirring every 5 minutes. The MBS-activated BSA was then purified by chromatography on a Bio-Gel P-10 column (Bio-Rad; 40 ml bed volume) equilibrated with 50 mM NaPO<sub>4</sub>, pH 7.0 buffer. Peak fractions were pooled (6.0 ml).

Lyophilized toxin A peptide (20 mg) was added to the activated BSA mixture, stirred until the peptide dissolved and incubated 3 hours at room temperature. Within 20 minutes, the reaction mixture became cloudy and precipitates formed. After 3 hours, the reaction mixture was centrifuged at  $10,000 \times g$  for 10 min and the supernatant analyzed for protein content. No significant protein could be detected at 280 nm. The conjugate precipitate was washed three times with PBS and stored at 4°C. A second conjugation was performed with 15 mg of activated BSA and 5 mg of peptide and the conjugates pooled and suspended at a peptide concentration of 10 mg/ml in 10 mM NaPO<sub>4</sub>, pH 7.2.

**b) Immunization Of Hens With Peptide Conjugate**

Two hens were each initially immunized on day zero by injection into two subcutaneous and two intramuscular sites with 1 mg of peptide conjugate that was emulsified in CFA (GIBCO). The hens were boosted on day 14 and day 21 with 1 mg of peptide conjugate emulsified in IFA (GIBCO).

**c) Detection Of Antitoxin Peptide Antibodies By ELISA**

IgY was purified from two eggs obtained before immunization (pre-immune) and two eggs obtained 31 and 32 days after the initial immunization using PEG fractionation as described in Example 1.

Wells of a 96-well microtiter plate (Falcon Pro-Bind Assay Plate) were coated overnight at 4°C with 100 µg/ml solution of the toxin A synthetic peptide in PBS, pH 7.2 prepared by dissolving 1 mg of the peptide in 1.0 ml of H<sub>2</sub>O and dilution of PBS. The pre-immune and immune IgY preparations were diluted in a five-fold series in a buffer containing 1% PEG 8000 and 0.1% Tween-20 (v/v) in PBS, pH 7.2. The wells were blocked for 2 hours at room temperature with 150 µl of a solution containing 5% (v/v) Carnation® nonfat dry milk and 1% PEG 8000 in PBS, pH 7.2. After incubation for 2 hours at room temperature, the wells were washed, secondary rabbit anti-chicken IgG-alkaline phosphatase (1:750) added, the wells washed again and the

color development obtained as described in Example 1. The results are shown in Table 12.

**TABLE 12**

Reactivity Of IgY With Toxin Peptide

Dilution Of PEG Prep	Absorbance At 410 nm	
	Preimmune	Immune Anti-Peptide
1:100	0.013	0.253
1:500	0.004	0.039
1:2500	0.004	0.005

Clearly, the immune antibodies contain titers against this repeated epitope of toxin A.

### EXAMPLE 8

#### Production Of Avian Antitoxins Against *Clostridium difficile* Native Toxins A And B

To determine whether avian antibodies are effective for the neutralization of *C. difficile* toxins, hens were immunized using native *C. difficile* toxins A and B. The resulting egg yolk antibodies were then extracted and assessed for their ability to neutralize toxins A and B *in vitro*. The Example involved (a) preparation of the toxin immunogens, (b) immunization, (c) purification of the antitoxins, and (d) assay of toxin neutralization activity.

#### a) Preparation Of The Toxin Immunogens

Both *C. difficile* native toxins A and B, and *C. difficile* toxoids, prepared by the treatment of the native toxins with formaldehyde, were employed as immunogens. *C. difficile* toxoids A and B were prepared by a procedure which was modified from published methods (Ehrich *et al.*, Infect. Immun. 28:1041 (1980). Separate solutions (in PBS) of native *C. difficile* toxin A and toxin B (Tech Lab) were each adjusted to a

concentration of 0.20 mg/ml, and formaldehyde was added to a final concentration of 0.4%. The toxin/formaldehyde solutions were then incubated at 37°C for 40 hrs. Free formaldehyde was then removed from the resulting toxoid solutions by dialysis against PBS at 4°C. In previously published reports, this dialysis step was not performed.

5 Therefore, free formaldehyde must have been present in their toxoid preparations. The toxoid solutions were concentrated, using a Centriprep concentrator unit (Amicon), to a final toxoid concentration of 4.0 mg/ml. The two resulting preparations were designated as toxoid A and toxoid B.

10 *C. difficile* native toxins were prepared by concentrating stock solutions of toxin A and toxin B (Tech Lab, Inc), using Centriprep concentrator units (Amicon), to a final concentration of 4.0 mg/ml.

#### b) Immunization

15 The first two immunizations were performed using the toxoid A and toxoid B immunogens described above. A total of 3 different immunization combinations were employed. For the first immunization group, 0.2 ml of toxoid A was emulsified in an equal volume of Titer Max adjuvant (CytRx). Titer Max was used in order to conserve the amount of immunogen used, and to simplify the immunization procedure. This immunization group was designated "CTA." For the second immunization group, 0.1 ml of toxoid B was emulsified in an equal volume of Titer Max adjuvant. This group was designated "CTB." For the third immunization group, 0.2 ml of toxoid A was first mixed with 0.2 ml of toxoid B, and the resulting mixture was emulsified in 0.4 ml of Titer Max adjuvant. This group was designated "CTAB." In this way, three separate immunogen emulsions were prepared, with each emulsion containing a final concentration of 2.0 mg/ml of toxoid A (CTA) or toxoid B (CTB) or a mixture of 2.0 mg/ml toxoid A and 2.0 mg/ml toxoid B (CTAB).

25 On day 0, White Leghorn hens, obtained from a local breeder, were immunized as follows: **Group CTA.** Four hens were immunized, with each hen receiving 200µg of toxoid A, via two intramuscular (I.M.) injections of 50µl of CTA emulsion in the breast area. **Group CTB.** One hen was immunized with 200µg of toxoid B, via two

I.M. injections of 50µl of CTB emulsion in the breast area. **Group CTAB.** Four  
hens were immunized, with each hen receiving a mixture containing 200µg of toxoid  
A and 200µg of toxoid B, via two I.M. injections of 100µl of CTAB emulsion in the  
breast area. The second immunization was performed 5 weeks later, on day 35,  
5 exactly as described for the first immunization above.

In order to determine whether hens previously immunized with *C. difficile*  
toxoids could tolerate subsequent booster immunizations using native toxins, a single  
hen from group CTAB was immunized for a third time, this time using a mixture of  
the native toxin A and native toxin B described in section (a) above (these toxins were  
10 not formaldehyde-treated, and were used in their active form). This was done in order  
to increase the amount (titer) and affinity of specific antitoxin antibody produced by  
the hen over that achieved by immunizing with toxoids only. On day 62, 0.1 ml of a  
toxin mixture was prepared which contained 200µg of native toxin A and 200µg of  
native toxin B. This toxin mixture was then emulsified in 0.1 ml of Titer Max  
15 adjuvant. A single CTAB hen was then immunized with the resulting immunogen  
emulsion, via two I.M. injections of 100µl each, into the breast area. This hen was  
marked with a wing band, and observed for adverse effects for a period of  
approximately 1 week, after which time the hen appeared to be in good health.

Because the CTAB hen described above tolerated the booster immunization  
20 with native toxins A and B with no adverse effects, it was decided to boost the  
remaining hens with native toxin as well. On day 70, booster immunizations were  
performed as follows: **Group CTA.** A 0.2 ml volume of the 4 mg/ml native toxin A  
solution was emulsified in an equal volume of Titer Max adjuvant. Each of the 4 hens  
was then immunized with 200µg of native toxin A, as described for the toxoid A  
25 immunizations above. **Group CTB.** A 50µl volume of the 4 mg/ml native toxin B  
solution was emulsified in an equal volume of Titer Max adjuvant. The hen was then  
immunized with 200µg of native toxin B, as described for the toxoid B immunizations  
above. **Group CTAB.** A 0.15 ml volume of the 4 mg/ml native toxin A solution was  
first mixed with a 0.15 ml volume the 4 mg/ml native toxin B solution. The resulting  
30 toxin mixture was then emulsified in 0.3 ml of Titer Max adjuvant. The 3 remaining

5 hens (the hen with the wing band was not immunized this time) were then immunized with 200µg of native toxin A and 200µg of native toxin B as described for the toxoid A+ toxoid B immunizations (CTAB) above. On day 85, all hens received a second booster immunization using native toxins, done exactly as described for the first boost with native toxins above.

All hens tolerated both booster immunizations with native toxins with no adverse effects. As previous literature references describe the use of formaldehyde-treated toxoids, this is apparently the first time that any immunizations have been performed using native *C. difficile* toxins.

#### 10 c) Purification Of Antitoxins

Eggs were collected from the hen in group CTB 10-12 days following the second immunization with toxoid (day 35 immunization described in section (b) above), and from the hens in groups CTA and CTAB 20-21 days following the second immunization with toxoid. To be used as a pre-immune (negative) control, eggs were  
15 also collected from unimmunized hens from the same flock. Egg yolk immunoglobulin (IgY) was extracted from the 4 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in the original yolk volume of PBS without thimerosal. Importantly, thimerosal was excluded because it would have been toxic to the CHO cells used in the toxin neutralization assays described in  
20 section (d) below.

#### d) Assay Of Toxin Neutralization Activity

The toxin neutralization activity of the IgY solutions prepared in section (c) above was determined using an assay system that was modified from published methods. [Ehrich *et al.*, Infect. Immun. 28:1041-1043 (1992); and McGee *et al.*  
25 Microb. Path. 12:333-341 (1992).] As additional controls, affinity-purified goat anti-*C. difficile* toxin A (Tech Lab) and affinity-purified goat anti-*C. difficile* toxin B (Tech Lab) were also assayed for toxin neutralization activity.



The IgY solutions and goat antibodies were serially diluted using F 12 medium (GIBCO) which was supplemented with 2% FCS (GIBCO)(this solution will be referred to as "medium" for the remainder of this Example). The resulting antibody solutions were then mixed with a standardized concentration of either native *C. difficile* toxin A (Tech Lab), or native *C. difficile* toxin B (Tech Lab), at the concentrations indicated below. Following incubation at 37°C for 60 min., 100µl volumes of the toxin + antibody mixtures were added to the wells of 96-well microtiter plates (Falcon Microtest III) which contained  $2.5 \times 10^4$  Chinese Hamster Ovary (CHO) cells per well (the CHO cells were plated on the previous day to allow them to adhere to the plate wells). The final concentration of toxin, or dilution of antibody indicated below refers to the final test concentration of each reagent present in the respective microtiter plate wells. Toxin reference wells were prepared which contained CHO cells and toxin A or toxin B at the same concentration used for the toxin plus antibody mixtures (these wells contained no antibody). Separate control wells were also prepared which contained CHO cells and medium only. The assay plates were then incubated for 18-24 hrs. in a 37°C, humidified, 5% CO<sub>2</sub> incubator. On the following day, the remaining adherent (viable) cells in the plate wells were stained using 0.2% crystal violet (Mallinckrodt) dissolved in 2% ethanol, for 10 min. Excess stain was then removed by rinsing with water, and the stained cells were solubilized by adding 100µl of 1% SDS (dissolved in water) to each well. The absorbance of each well was then measured at 570 nm, and the percent cytotoxicity of each test sample or mixture was calculated using the following formula:

$$\% \text{ CHO Cell Cytotoxicity} = [1 - (\frac{\text{Abs. Sample}}{\text{Abs. Control}})] \times 100$$

Unlike previous reports which quantitate results visually by counting cell rounding by microscopy, this Example utilized spectrophotometric methods to quantitate the *C. difficile* toxin bioassay. In order to determine the toxin A

neutralizing activity of the CTA, CTAB, and pre-immune IgY preparations, as well as the affinity-purified goat antitoxin A control, dilutions of these antibodies were reacted against a 0.1µg/ml concentration of native toxin A (this is the approx. 50% cytotoxic dose of toxin A in this assay system). The results are shown in Figure 3.

5 Complete neutralization of toxin A occurred with the CTA IgY (antitoxin A, above) at dilutions of 1:80 and lower, while significant neutralization occurred out to the 1:320 dilution. The CTAB IgY (antitoxin A + toxin B, above) demonstrated complete neutralization at the 1:320-1:160 and lower dilutions, and significant neutralization occurred out to the 1:1280 dilution. The commercially available  
10 affinity-purified goat antitoxin A did not completely neutralize toxin A at any of the dilutions tested, but demonstrated significant neutralization out to a dilution of 1:1,280. The preimmune IgY did not show any toxin A neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized with toxin A alone, or simultaneously with toxin A and toxin B, is an  
15 effective toxin A antitoxin.

The toxin B neutralizing activity of the CTAB and pre-immune IgY preparations, and also the affinity-purified goat antitoxin B control was determined by reacting dilutions of these antibodies against a concentration of native toxin B of 0.1 ng/ml (approximately the 50% cytotoxic dose of toxin B in the assay system). The  
20 results are shown in Figure 4.

Complete neutralization of toxin B occurred with the CTAB IgY (antitoxin A + toxin B, above) at the 1:40 and lower dilutions, and significant neutralization occurred out to the 1:320 dilution. The affinity-purified goat antitoxin B demonstrated complete neutralization at dilutions of 1:640 and lower, and significant neutralization occurred  
25 out to a dilution of 1:2,560. The preimmune IgY did not show any toxin B neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized simultaneously with toxin A and toxin B is an effective toxin B antitoxin.

In a separate study, the toxin B neutralizing activity of CTB, CTAB, and pre-immune IgY preparations was determined by reacting dilutions of these antibodies  
30

against a native toxin B concentration of 0.1 µg/ml (approximately 100% cytotoxic dose of toxin B in this assay system). The results are shown in Figure 5.

Significant neutralization of toxin B occurred with the CTB IgY (antitoxin B, above) at dilutions of 1:80 and lower, while the CTAB IgY (antitoxin A + toxin B, above) was found to have significant neutralizing activity at dilutions of 1:40 and lower. The preimmune IgY did not show any toxin B neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized with toxin B alone, or simultaneously with toxin A and toxin B, is an effective toxin B antitoxin.

### EXAMPLE 9

#### *In vivo* Protection Of Golden Syrian Hamsters From *C. difficile* Disease By Avian Antitoxins Against *C. difficile* Toxins A And B

The most extensively used animal model to study *C. difficile* disease is the hamster. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1992).] Several other animal models for antibiotic-induced diarrhea exist, but none mimic the human form of the disease as closely as the hamster model. [R. Fekety, "Animal Models of Antibiotic-Induced Colitis," in O. Zak and M. Sande (eds.), *Experimental Models in Antimicrobial Chemotherapy*, Vol. 2, pp.61-72, (1986).] In this model, the animals are first predisposed to the disease by the oral administration of an antibiotic, such as clindamycin, which alters the population of normally-occurring gastrointestinal flora (Fekety, at 61-72). Following the oral challenge of these animals with viable *C. difficile* organisms, the hamsters develop cecitis, and hemorrhage, ulceration, and inflammation are evident in the intestinal mucosa. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1985).] The animals become lethargic, develop severe diarrhea, and a high percentage of them die from the disease. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1985).] This model is therefore ideally suited for the evaluation of therapeutic agents designed for the treatment or prophylaxis of *C. difficile* disease.

The ability of the avian *C. difficile* antitoxins, described in Example 1 above, to protect hamsters from *C. difficile* disease was evaluated using the Golden Syrian hamster model of *C. difficile* infection. The Example involved (a) preparation of the avian *C. difficile* antitoxins, (b) *in vivo* protection of hamsters from *C. difficile* disease by treatment with avian antitoxins, and (c) long-term survival of treated hamsters.

**a) Preparation Of The Avian *C. difficile* Antitoxins**

Eggs were collected from hens in groups CTA and CTAB described in Example 1 (b) above. To be used as a pre-immune (negative) control, eggs were also purchased from a local supermarket. Egg yolk immunoglobulin (IgY) was extracted from the 3 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in one fourth the original yolk volume of Ensure® nutritional formula.

**b) *In vivo* Protection Of Hamsters Against *C. difficile*  
Disease By Treatment With Avian Antitoxins**

The avian *C. difficile* antitoxins prepared in section (a) above were evaluated for their ability to protect hamsters from *C. difficile* disease using an animal model system which was modified from published procedures. [Fekety, at 61-72; Borriello *et al.*, J. Med. Microbiol., 24:53-64 (1987); Kim *et al.*, Infect. Immun., 55:2984-2992 (1987); Borriello *et al.*, J. Med. Microbiol., 25:191-196 (1988); Delmee and Avesani, J. Med. Microbiol., 33:85-90 (1990); and Lysterly *et al.*, Infect. Immun., 59:2215-2218 (1991).] For the study, three separate experimental groups were used, with each group consisting of 7 female Golden Syrian hamsters (Charles River), approximately 10 weeks old and weighing approximately 100 gms. each. The three groups were designated "CTA," "CTAB" and "Pre-immune." These designations corresponded to the antitoxin preparations with which the animals in each group were treated. Each animal was housed in an individual cage, and was offered food and water *ad libitum* through the entire length of the study. On day 1, each animal was orally administered

1.0 ml of one of the three antitoxin preparations (prepared in section (a) above) at the following timepoints: 0 hrs., 4 hrs., and 8 hrs. On day 2, the day 1 treatment was repeated. On day 3, at the 0 hr. timepoint, each animal was again administered antitoxin, as described above. At 1 hr., each animal was orally administered 3.0 mg of clindamycin-HCl (Sigma) in 1 ml of water. This treatment predisposed the animals to infection with *C. difficile*. As a control for possible endogenous *C. difficile* colonization, an additional animal from the same shipment (untreated) was also administered 3.0 mg of clindamycin-HCl in the same manner. This clindamycin control animal was left untreated (and uninfected) for the remainder of the study. At the 4 hr. and 8 hr. timepoints, the animals were administered antitoxin as described above. On day 4, at the 0 hr. timepoint, each animal was again administered antitoxin as described above. At 1 hr., each animal was orally challenged with 1 ml of *C. difficile* inoculum, which contained approx. 100 *C. difficile* strain 43596 organisms in sterile saline. *C. difficile* strain 43596, which is a serogroup C strain, was chosen because it is representative of one of the most frequently-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee *et al.*, J. Clin. Microbiol., 28:2210-2214 (1985).] In addition, this strain has been previously demonstrated to be virulent in the hamster model of infection. [Delmee and Avesani, J. Med. Microbiol., 33:85-90 (1990).] At the 4 hr. and 8 hr. timepoints, the animals were administered antitoxin as described above. On days 5 through 13, the animals were administered antitoxin 3x per day as described for day 1 above, and observed for the onset of diarrhea and death. On the morning of day 14, the final results of the study were tabulated. These results are shown in Table 13.

Representative animals from those that died in the Pre-Immune and CTA groups were necropsied. Viable *C. difficile* organisms were cultured from the ceca of these animals, and the gross pathology of the gastrointestinal tracts of these animals was consistent with that expected for *C. difficile* disease (inflamed, distended, hemorrhagic cecum, filled with watery diarrhea-like material). In addition, the clindamycin control animal remained healthy throughout the entire study period,

therefore indicating that the hamsters used in the study had not previously been colonized with endogenous *C. difficile* organisms prior to the start of the study. Following the final antitoxin treatment on day 13, a single surviving animal from the CTA group, and also from the CTAB group, was sacrificed and necropsied. No pathology was noted in either animal.

TABLE 13

Treatment Results

Treatment Group	No. Animals Surviving	No. Animals Dead
Pre-Immune	1	6
CTA (Antitoxin A only)	5	2
CTAB (Antitoxin A + Antitoxin B)	7	0

Treatment of hamsters with orally-administered toxin A and toxin B antitoxin (group CTAB) successfully protected 7 out of 7 (100%) of the animals from *C. difficile* disease. Treatment of hamsters with orally-administered toxin A antitoxin (group CTA) protected 5 out of 7 (71%) of these animals from *C. difficile* disease. Treatment using pre-immune IgY was not protective against *C. difficile* disease, as only 1 out of 7 (14%) of these animals survived. These results demonstrate that the avian toxin A antitoxin and the avian toxin A + toxin B antitoxin effectively protected the hamsters from *C. difficile* disease. These results also suggest that although the neutralization of toxin A alone confers some degree of protection against *C. difficile* disease, in order to achieve maximal protection, simultaneous antitoxin A and antitoxin B activity is necessary.

#### c) Long-Term Survival Of Treated Hamsters

It has been previously reported in the literature that hamsters treated with orally-administered bovine antitoxin IgG concentrate are protected from *C. difficile* disease as long as the treatment is continued, but when the treatment is stopped, the animals develop diarrhea and subsequently die within 72 hrs. [Lyerly *et al.*, Infect. Immun., 59(6):2215-2218 (1991).]

In order to determine whether treatment of *C. difficile* disease using avian antitoxins promotes long-term survival following the discontinuation of treatment, the 4 surviving animals in group CTA, and the 6 surviving animals in group CTAB were observed for a period of 11 days (264 hrs.) following the discontinuation of antitoxin treatment described in section (b) above. All hamsters remained healthy through the entire post-treatment period. This result demonstrates that not only does treatment with avian antitoxin protect against the onset of *C. difficile* disease (i.e., it is effective as a prophylactic), it also promotes long-term survival beyond the treatment period, and thus provides a lasting cure.

#### EXAMPLE 10

##### *In vivo* Treatment Of Established *C. difficile* Infection In Golden Syrian Hamsters With Avian Antitoxins Against *C. difficile* Toxins A And B

The ability of the avian *C. difficile* antitoxins, described in Example 8 above, to treat an established *C. difficile* infection was evaluated using the Golden Syrian hamster model. The Example involved (a) preparation of the avian *C. difficile* antitoxins, (b) *in vivo* treatment of hamsters with established *C. difficile* infection, and (c) histologic evaluation of cecal tissue.

##### **a) Preparation Of The Avian *C. difficile* Antitoxins**

Eggs were collected from hens in group CTAB described in Example 8 (b) above, which were immunized with *C. difficile* toxoids and native toxins A and B. Eggs purchased from a local supermarket were used as a pre-immune (negative) control. Egg yolk immunoglobulin (IgY) was extracted from the 2 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in one-fourth the original yolk volume of Ensure® nutritional formula.

b) ***In vivo* Treatment Of Hamsters With Established  
*C. difficile* Infection**

The avian *C. difficile* antitoxins prepared in section (a) above were evaluated for the ability to treat established *C. difficile* infection in hamsters using an animal model system which was modified from the procedure which was described for the hamster protection study in Example 8(b) above.

For the study, four separate experimental groups were used, with each group consisting of 7 female Golden Syrian hamsters (Charles River), approx. 10 weeks old, weighing approximately 100 gms. each. Each animal was housed separately, and was offered food and water *ad libitum* through the entire length of the study.

On day 1 of the study, the animals in all four groups were each predisposed to *C. difficile* infection by the oral administration of 3.0 mg of clindamycin-HCl (Sigma) in 1 ml of water.

On day 2, each animal in all four groups was orally challenged with 1 ml of *C. difficile* inoculum, which contained approximately 100 *C. difficile* strain 43596 organisms in sterile saline. *C. difficile* strain 43596 was chosen because it is representative of one of the most frequently-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee *et al.*, J. Clin. Microbiol., 28:2210-2214 (1990).] In addition, as this was the same *C. difficile* strain used in all of the previous Examples above, it was again used in order to provide experimental continuity.

On day 3 of the study (24 hrs. post-infection), treatment was started for two of the four groups of animals. Each animal of one group was orally administered 1.0 ml of the CTAB IgY preparation (prepared in section (a) above) at the following timepoints: 0 hrs., 4 hrs., and 8 hrs. The animals in this group were designated "CTAB-24." The animals in the second group were each orally administered 1.0 ml of the pre-immune IgY preparation (also prepared in section (a) above) at the same timepoints as for the CTAB group. These animals were designated "Pre-24." Nothing was done to the remaining two groups of animals on day 3.



On day 4, 48 hrs. post-infection, the treatment described for day 3 above was repeated for the CTAB-24 and Pre-24 groups, and was initiated for the remaining two groups at the same timepoints. The final two groups of animals were designated "CTAB-48" and "Pre-48" respectively.

5 On days 5 through 9, the animals in all four groups were administered antitoxin or pre-immune IgY, 3x per day, as described for day 4 above. The four experimental groups are summarized in Table 14.

**TABLE 14**

Experimental Treatment Groups

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Group Designation	Experimental Treatment
CTAB-24	Infected, treatment w/antitoxin IgY started @ 24 hrs. post-infection.
Pre-24	Infected, treatment w/pre-immune IgY started @ 24 hrs. post-infection.
CTAB-48	Infected, treatment w/antitoxin IgY started @ 48 hrs. post-infection.
Pre-48	Infected, treatment w/pre-immune IgY started @ 48 hrs. post-infection.

15 All animals were observed for the onset of diarrhea and death through the conclusion of the study on the morning of day 10. The results of this study are displayed in Table 15.

**TABLE 15**

Experimental Outcome--Day 10

20

Treatment Group	No. Animals Surviving	No. Animals Dead
CTAB-24	6	1
Pre-24	0	7
CTAB-48	4	3
Pre-48	2	5

25 Eighty-six percent of the animals which began receiving treatment with antitoxin IgY at 24 hrs. post-infection (CTAB-24 above) survived, while 57% of the animals treated with antitoxin IgY starting 48 hrs. post-infection (CTAB-48 above) survived. In contrast, none of the animals receiving pre-immune IgY starting 24 hrs.

post-infection (Pre-24 above) survived, and only 29% of the animals which began receiving treatment with pre-immune IgY at 48 hrs. post-infection (Pre-48 above) survived through the conclusion of the study. These results demonstrate that avian antitoxins raised against *C. difficile* toxins A and B are capable of successfully treating established *C. difficile* infections *in vivo*.

### c) Histologic Evaluation Of Cecal Tissue

In order to further evaluate the ability of the IgY preparations tested in this study to treat established *C. difficile* infection, histologic evaluations were performed on cecal tissue specimens obtained from representative animals from the study described in section (b) above.

Immediately following death, cecal tissue specimens were removed from animals which died in the Pre-24 and Pre-48 groups. Following the completion of the study, a representative surviving animal was sacrificed and cecal tissue specimens were removed from the CTAB-24 and CTAB-48 groups. A single untreated animal from the same shipment as those used in the study was also sacrificed and a cecal tissue specimen was removed as a normal control. All tissue specimens were fixed overnight at 4°C in 10% buffered formalin. The fixed tissues were paraffin-embedded, sectioned, and mounted on glass microscope slides. The tissue sections were then stained using hematoxylin and eosin (H and E stain), and were examined by light microscopy.

Upon examination, the tissues obtained from the CTAB-24 and CTAB-48 animals showed no pathology, and were indistinguishable from the normal control. This observation provides further evidence for the ability of avian antitoxins raised against *C. difficile* toxins A and B to effectively treat established *C. difficile* infection, and to prevent the pathologic consequences which normally occur as a result of *C. difficile* disease.

In contrast, characteristic substantial mucosal damage and destruction was observed in the tissues of the animals from the Pre-24 and Pre-48 groups which died

from *C. difficile* disease. Normal tissue architecture was obliterated in these two preparations, as most of the mucosal layer was observed to have sloughed away, and there were numerous large hemorrhagic areas containing massive numbers of erythrocytes.

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## EXAMPLE 11

### Cloning And Expression Of *C. difficile* Toxin A Fragments

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The toxin A gene has been cloned and sequenced, and shown to encode a protein of predicted MW of 308 kd. [Dove *et al.*, Infect. Immun., 58:480-488 (1990).] Given the expense and difficulty of isolating native toxin A protein, it would be advantageous to use simple and inexpensive procaryotic expression systems to produce and purify high levels of recombinant toxin A protein for immunization purposes. Ideally, the isolated recombinant protein would be soluble in order to preserve native antigenicity, since solubilized inclusion body proteins often do not fold into native conformations. To allow ease of purification, the recombinant protein should be expressed to levels greater than 1 mg/liter of *E. coli* culture.

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To determine whether high levels of recombinant toxin A protein can be produced in *E. coli*, fragments of the toxin A gene were cloned into various prokaryotic expression vectors, and assessed for the ability to express recombinant toxin A protein in *E. coli*. Three prokaryotic expression systems were utilized. These systems were chosen because they drive expression of either fusion (pMALc and pGEX2T) or native (pET23a-c) protein to high levels in *E. coli*, and allow affinity purification of the expressed protein on a ligand containing column. Fusion proteins expressed from pGEX vectors bind glutathione agarose beads, and are eluted with reduced glutathione. pMAL fusion proteins bind amylose resin, and are eluted with maltose. A poly-histidine tag is present at either the N-terminal (pET16b) or C-terminal (pET23a-c) end of pET fusion proteins. This sequence specifically binds  $\text{Ni}_2^+$  chelate columns, and is eluted with imidazole salts. Extensive descriptions of these

vectors are available [Williams *et al.* (1995) *DNA Cloning 2: Expression Systems*, Glover and Hames, eds. IRL Press, Oxford, pp. 15-58], and will not be discussed in detail here. The Example involved (a) cloning of the toxin A gene, (b) expression of large fragments of toxin A in various prokaryotic expression systems, (c) identification of smaller toxin A gene fragments that express efficiently in *E. coli*, (d) purification of recombinant toxin A protein by affinity chromatography, and (e) demonstration of functional activity of a recombinant fragment of the toxin A gene.

#### a) Cloning Of The Toxin A Gene

A restriction map of the toxin A gene is shown in Figure 6. The encoded protein contains a carboxy terminal ligand binding region, containing multiple repeats of a carbohydrate binding domain. [von Eichel-Streiber and Sauerborn, *Gene* 96:107-113 (1990).] The toxin A gene was cloned in three pieces, by using either the polymerase chain reaction (PCR) to amplify specific regions, (regions 1 and 2, Figure 6) or by screening a constructed genomic library for a specific toxin A gene fragment (region 3, Figure 6). The sequences of the utilized PCR primers are P1: 5' GGAAATT TAGCTGCAGCATCTGAC 3' (SEQ ID NO.:1); P2: 5' TCTAGCAAATTCGCTTGT GTTGAA 3' (SEQ ID NO.:2); P3: 5' CTCGCATATAGCATTAGACC 3' (SEQ ID NO.:3); and P4: 5' CTATCTAGGCCTAAAGTAT 3' (SEQ ID NO.:4). These regions were cloned into prokaryotic expression vectors that express either fusion (pMALc and pGEX2T) or native (pET23a-c) protein to high levels in *E. coli*, and allow affinity purification of the expressed protein on a ligand containing column.

*Clostridium difficile* VPI strain 10463 was obtained from the ATCC (ATCC #43255) and grown under anaerobic conditions in brain-heart infusion medium (BBL). High molecular-weight *C. difficile* DNA was isolated essentially as described by Wren and Tabaqchali (1987) *J. Clin. Microbiol.*, 25:2402, except proteinase K and sodium dodecyl sulfate (SDS) was used to disrupt the bacteria, and cetyltrimethylammonium bromide precipitation [as described in Ausubel *et al.*, *Current Protocols in Molecular Biology* (1989)] was used to remove carbohydrates from the cleared lysate. The

integrity and yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

Fragments 1 and 2 were cloned by PCR, utilizing a proofreading thermostable DNA polymerase (native *pfu* polymerase; Stratagene). The high fidelity of this  
5 polymerase reduces the mutation problems associated with amplification by error prone polymerases (*e.g.*, *Taq* polymerase). PCR amplification was performed using the indicated PCR primers (Figure 6) in 50 µl reactions containing 10 mM Tris-HCl(8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM each dNTP, 0.2 µM each primer, and 50 ng *C. difficile* genomic DNA. Reactions were overlaid with 100 µl mineral oil, heated to  
10 94°C for 4 min, 0.5 µl native *pfu* polymerase (Stratagene) added, and the reaction cycled 30x at 94°C for 1 min, 50°C for 1 min, 72°C for 4 min, followed by 10 min at 72°C. Duplicate reactions were pooled, chloroform extracted, and ethanol precipitated. After washing in 70% ethanol, the pellets were resuspended in 50 µl TE buffer [10 mM Tris-HCL, 1 mM EDTA pH 8.0]. Aliquots of 10µl each were restriction digested  
15 with either *EcoRI/HincII* (fragment 1) or *EcoRI/PstI* (fragment 2), and the appropriate restriction fragments were gel purified using the Prep-A-Gene kit (BioRad), and ligated to either *EcoRI/SmaI*-restricted pGEX2T (Pharmacia) vector (fragment 1), or the *EcoRI/PstI* pMALc (New England Biolabs) vector (fragment 2). Both clones are predicted to produce in-frame fusions with either the glutathione-S-transferase protein (pGEX vector) or the maltose binding protein (pMAL vector). Recombinant clones  
20 were isolated, and confirmed by restriction digestion, using standard recombinant molecular biology techniques. [Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (1989), and designated pGA30-660 and pMA660-1100, respectively (*see* Figure 6 for description of the clone designations).]

25 Fragment 3 was cloned from a genomic library of size selected *PstI* digested *C. difficile* genomic DNA, using standard molecular biology techniques (Sambrook *et al.*). Given that the fragment 3 internal *PstI* site is protected from cleavage in *C. difficile* genomic DNA [Price *et al.*, *Curr. Microbiol.*, 16:55-60 (1987)], a 4.7 kb fragment from *PstI* restricted *C. difficile* genomic DNA was gel purified, and ligated to

*Pst*I restricted, phosphatase treated pUC9 DNA. The resulting genomic library was screened with a oligonucleotide primer specific to fragment 3, and multiple independent clones were isolated. The presence of fragment 3 in several of these clones was confirmed by restriction digestion, and a clone of the indicated orientation (Figure 6) was restricted with *Bam*HI/*Hind*III, the released fragment purified by gel electrophoresis, and ligated into similarly restricted pET23c expression vector DNA (Novagen). Recombinant clones were isolated, and confirmed by restriction digestion. This construct is predicted to create both a predicted in frame fusion with the pET protein leader sequence, as well as a predicted C-terminal poly-histidine affinity tag, and is designated pPA1100-2680 (see Figure 6 for the clone designation).

#### **b) Expression Of Large Fragments Of Toxin A In *E. coli***

Protein expression from the three expression constructs made in (a) was induced, and analyzed by Western blot analysis with an affinity purified, goat polyclonal antiserum directed against the toxin A toxoid (Tech Lab). The procedures utilized for protein induction, SDS-PAGE, and Western blot analysis are described in detail in Williams *et al* (1995), *supra*. In brief, 5 ml 2X YT (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter, pH 7.5 + 100 µg/ml ampicillin were added to cultures of bacteria (BL21 for pMA1 and pGEX plasmids, and BL21(DE3)LysS for pET plasmids) containing the appropriate recombinant clone which were induced to express recombinant protein by addition of IPTG to 1 mM. Cultures were grown at 37°C, and induced when the cell density reached 0.5 OD<sub>600</sub>. Induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in a microfuge), and resuspension of the pelleted bacteria in 150 µl of 2x SDS-PAGE sample buffer [Williams *et al.* (1995), *supra*]. The samples were heated to 95°C for 5 min, the cooled and 5 or 10 µl aliquots loaded on 7.5% SDS-PAGE gels. BioRad high molecular weight protein markers were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining gels with Coomassie

blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. Western blots, (performed as described in Example 3) which detect toxin A reactive protein in cell lysates of induced protein from the three expression constructs are shown in Figure 7. In this figure, lanes 1-3 contain cell  
5 lysates prepared from *E. coli* strains containing pPA1100-2860 in B121(DE3)lysE cells; lanes 4-6 contain cell lysates prepared from *E. coli* strains containing pPA1100-2860 in B121(DE3)lysS cells; lanes 7-9 contain cell lysates prepared from *E. coli* strains containing pMA30-660; lanes 10-12 contain cell lysates prepared from *E. coli* strains containing pMA660-1100. The lanes were probed with an affinity purified goat  
10 antitoxin A polyclonal antibody (Tech Lab). Control lysates from uninduced cells (lanes 1, 7, and 10) contain very little immunoreactive material compared to the induced samples in the remaining lanes. The highest molecular weight band observed for each clone is consistent with the predicted size of the full length fusion protein.

Each construct directs expression of high molecular weight (HMW) protein that  
15 is reactive with the toxin A antibody. The size of the largest immunoreactive bands from each sample is consistent with predictions of the estimated MW of the intact fusion proteins. This demonstrates that the three fusions are in-frame, and that none of the clones contain cloning artifacts that disrupt the integrity of the encoded fusion protein. However, the Western blot demonstrates that fusion protein from the two  
20 larger constructs (pGA30-660 and pPA1100-2680) are highly degraded. Also, expression levels of toxin A proteins from these two constructs are low, since induced protein bands are not visible by Coomassie staining (not shown). Several other expression constructs that fuse large sub-regions of the toxin A gene to either pMALc or pET23a-c expression vectors, were constructed and tested for protein induction.  
25 These constructs were made by mixing gel purified restriction fragments, derived from the expression constructs shown in Figure 6, with appropriately cleaved expression vectors, ligating, and selecting recombinant clones in which the toxin A restriction fragments had ligated together and into the expression vector as predicted for in-frame fusions. The expressed toxin A interval within these constructs are shown in Figure 8,  
30 as well as the internal restriction sites utilized to make these constructs.

As used herein, the term "interval" refers to any portion (*i.e.*, any segment of the toxin which is less than the whole toxin molecule) of a clostridial toxin. In a preferred embodiment, "interval" refers to portions of *C. difficile* toxins such as toxin A or toxin B. It is also contemplated that these intervals will correspond to epitopes of immunologic importance, such as antigens or immunogens against which a neutralizing antibody response is effected. It is not intended that the present invention be limited to the particular intervals or sequences described in these Examples. It is also contemplated that sub-portions of intervals (*e.g.*, an epitope contained within one interval or which bridges multiple intervals) be used as compositions and in the methods of the present invention.

In all cases, Western blot analysis of each of these constructs with goat antitoxin A antibody (Tech Lab) detected HMW fusion protein of the predicted size (not shown). This confirms that the reading frame of each of these clones is not prematurely terminated, and is fused in the correct frame with the fusion partner. However, the Western blot analysis revealed that in all cases, the induced protein is highly degraded, and, as assessed by the absence of identifiable induced protein bands by Coomassie Blue staining, are expressed only at low levels. These results suggest that expression of high levels of intact toxin A recombinant protein is not possible when large regions of the toxin A gene are expressed in *E. coli* using these expression vectors.

**c) High Level Expression Of Small Toxin A Protein  
Fusions In *E. coli***

Experience indicates that expression difficulties are often encountered when large (greater than 100 kd) fragments are expressed in *E. coli*. A number of expression constructs containing smaller fragments of the toxin A gene were constructed, to determine if small regions of the gene can be expressed to high levels without extensive protein degradation. A summary of these expression constructs are shown in Figure 9. All were constructed by in-frame fusions of convenient toxin A



restriction fragments to either the pMALc or pET23a-c vectors. Protein preparations from induced cultures of each of these constructs were analyzed by both Coomassie Blue staining and Western analysis as in (b) above. In all cases, higher levels of intact, full length fusion proteins were observed than with the larger recombinants from section (b).

**d) Purification Of Recombinant Toxin A Protein**

Large scale (500 ml) cultures of each recombinant from (c) were grown, induced, and soluble and insoluble protein fractions were isolated. The soluble protein extracts were affinity chromatographed to isolate recombinant fusion protein, as described [Williams *et al.* (1994), *supra*]. In brief, extracts containing tagged pET fusions were chromatographed on a nickel chelate column, and eluted using imidazole salts as described by the distributor (Novagen). Extracts containing soluble pMAL fusion protein were prepared and chromatographed in column buffer (10 mM NaPO<sub>4</sub>, 0.5M NaCl, 10 mM β-mercaptoethanol, pH 7.2) over an amylose resin column (New England Biolabs), and eluted with column buffer containing 10 mM maltose as described [Williams *et al.* (1995), *supra*]. When the expressed protein was found to be predominantly insoluble, insoluble protein extracts were prepared by the method described in Example 17, *infra*. The results are summarized in Table 16. Figure 10 shows the sample purifications of recombinant toxin A protein. In this figure, lanes 1 and 2 contain MBP fusion protein purified by affinity purification of soluble protein.

TABLE 16

## Purification Of Recombinant Toxin A Protein

Clone <sup>(a)</sup>	Protein Solubility	Yield Affinity Purified Soluble Protein <sup>(b)</sup>	% Intact Soluble Fusion Protein <sup>(c)</sup>	Yield Intact Insoluble Fusion Protein
pMA30-270	Soluble	4 mg/500 mls	10%	NA
PMA30-300	Soluble	4 mg/500 mls	5-10%	NA
pMA300-660	Insoluble	—	NA	10 mg/500 ml
pMA660-1100	Soluble	4.5 mg/500 mls	50%	NA
pMA1100-1610	Soluble	18 mg/500 mls	10%	NA
pMA1610-1870	Both	22 mg/500 mls	90%	20 mg/500 ml
pMA1450-1870	Insoluble	-----	NA	0.2 mg/500 ml
pPA1100-1450	Soluble	0.1 mg/500 mls	90%	NA
pPA1100-1870	Soluble	0.02 mg/500 mls	90%	NA
pMA1870-2680	Both	12 mg/500 mls	80%	NA
pPa1870-2680	Insoluble	-----	NA	10 mg/500 ml

<sup>(a)</sup> pP = pET23 vector, pM=pMALc vector, A=toxin A.

<sup>(b)</sup> Based on  $1.5 \text{ OD}_{280} = 1 \text{ mg/ml}$  (extinction coefficient of MBP).

<sup>(c)</sup> Estimated by Coomassie staining of SDS-PAGE gels.

Lanes 3 and 4 contain MBP fusion protein purified by solubilization of insoluble inclusion bodies. The purified fusion protein samples are pMA1870-2680 (lane 1), pMA660-1100 (lane 2), pMA300-600 (lane 3) and pMA1450-1870 (lane 4).

Poor yields of affinity purified protein were obtained when poly-histidine tagged pET vectors were used to drive expression (pPA1100-1450, pP1100-1870). However, significant protein yields were obtained from pMAL expression constructs spanning the entire toxin A gene, and yields of full-length soluble fusion protein ranged from an estimated 200-400  $\mu\text{g}/500 \text{ ml}$  culture (pMA30-300) to greater than 20 mg/500 ml culture (pMA1610-1870). Only one interval was expressed to high levels as strictly insoluble protein (pMA300-660). Thus, although high level expression was not observed when using large expression constructs from the toxin A gene, usable levels of recombinant protein spanning the entire toxin A gene were obtainable by

isolating induced protein from a series of smaller pMAL expression constructs that span the entire toxin A gene. This is the first demonstration of the feasibility of expressing recombinant toxin A protein to high levels in *E. coli*.

e) **Hemagglutination Assay Using The Toxin A  
Recombinant Proteins**

The carboxy terminal end consisting of the repeating units contains the hemagglutination activity or binding domain of *C. difficile* toxin A. To determine whether the expressed toxin A recombinants retain functional activity, hemagglutination assays were performed. Two toxin A recombinant proteins, one containing the binding domain as either soluble affinity purified protein (pMA1870-2680) or SDS solubilized inclusion body protein (pPA1870-2680) and soluble protein from one region outside that domain (pMA1100-1610) were tested using a described procedure. [H.C. Krivan *et. al.*, Infect. Immun., 53:573 (1986).] Citrated rabbit red blood cells (RRBC)(Cocalico) were washed several times with Tris-buffer ( 0.1M Tris and 50 mM NaCl ) by centrifugation at 450 x g for 10 minutes at 4° C. A 1% RRBC suspension was made from the packed cells and resuspended in Tris-buffer. Dilutions of the recombinant proteins and native toxin A (Tech Labs) were made in the Tris-buffer and added in duplicate to a round-bottomed 96-well microtiter plate in a final volume of 100 µl. To each well, 50 µl of the 1% RRBC suspension was added, mixed by gentle tapping, and incubated at 4°C for 3-4 hours. Significant hemagglutination occurred only in the recombinant proteins containing the binding domain (pMA 1870-2680) and native toxin A. The recombinant protein outside the binding domain (pMA 1100-1610) displayed no hemagglutination activity. Using equivalent protein concentrations, the hemagglutination titer for toxin A was 1:256, while titers for the soluble and insoluble recombinant proteins of the binding domain were 1:256 and about 1:5000. Clearly, the recombinant proteins tested retained functional activity and were able to bind RRBC's.

## EXAMPLE 12

### Functional Activity Of IgY Reactive Against Toxin A Recombinants

The expression of recombinant toxin A protein as multiple fragments in *E.coli* has demonstrated the feasibility of generating toxin A antigen through use of recombinant methodologies (Example 11). The isolation of these recombinant proteins allows the immunoreactivity of each individual subregion of the toxin A protein to be determined (*i.e.*, in a antibody pool directed against the native toxin A protein). This identifies the regions (if any) for which little or no antibody response is elicited when the whole protein is used as a immunogen. Antibodies directed against specific fragments of the toxin A protein can be purified by affinity chromatography against recombinant toxin A protein, and tested for neutralization ability. This identifies any toxin A subregions that are essential for producing neutralizing antibodies. Comparison with the levels of immune response directed against these intervals when native toxin is used as an immunogen predicts whether potentially higher titers of neutralizing antibodies can be produced by using recombinant protein directed against a individual region, rather than the entire protein. Finally, since it is unknown whether antibodies reactive to the recombinant toxin A proteins produced in Example 11 neutralize toxin A as effectively as antibodies raised against native toxin A (Examples 9 and 10), the protective ability of a pool of antibodies affinity purified against recombinant toxin A fragments was assessed for its ability to neutralize toxin A.

This Example involved (a) epitope mapping of the toxin A protein to determine the titre of specific antibodies directed against individual subregions of the toxin A protein when native toxin A protein is used as an immunogen, (b) affinity purification of IgY reactive against recombinant proteins spanning the toxin A gene, (c) toxin A neutralization assays with affinity purified IgY reactive to recombinant toxin A protein to identify subregions of the toxin A protein that induce the production of neutralizing antibodies, and determination of whether complete neutralization of toxin A can be elicited with a mixture of antibodies reactive to recombinant toxin A protein.

a) **Epitope Mapping Of The Toxin A Gene**

The affinity purification of recombinant toxin A protein specific to defined intervals of the toxin A protein allows epitope mapping of antibody pools directed against native toxin A. This has not previously been possible, since previous  
5 expression of toxin A recombinants has been assessed only by Western blot analysis, without knowledge of the expression levels of the protein [e.g., von Eichel-Streiber *et al.*, J. Gen. Microbiol., 135:55-64 (1989)]. Thus, high or low reactivity of recombinant toxin A protein on Western blots may reflect protein expression level differences, not immunoreactivity differences. Given that the purified recombinant protein generated in  
10 Example 11 have been quantitated, the issue of relative immunoreactivity of individual regions of the toxin A protein was precisely addressed.

For the purposes of this Example, the toxin A protein was subdivided into 6 intervals (1-6), numbered from the amino (interval 1) to the carboxyl (interval 6) termini.

15 The recombinant proteins corresponding to these intervals were from expression clones (see Example 11(d) for clone designations) pMA30-300 (interval 1), pMA300-660 (interval 2), pMA660-1100 (interval 3), pPA1100-1450 (interval 4), pMA1450-1870 (interval 5) and pMA1870-2680 (interval 6). These 6 clones were selected because they span the entire protein from amino acids numbered 30 through 2680, and  
20 subdivide the protein into 6 small intervals. Also, the carbohydrate binding repeat interval is contained specifically in one interval (interval 6), allowing evaluation of the immune response specifically directed against this region. Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of each recombinant protein, were probed with either goat antitoxin A polyclonal antibody  
25 (Tech Lab) or chicken antitoxin A polyclonal antibody [pCTA IgY, Example 8(c)]. The blots were prepared and developed with alkaline phosphatase as previously described [Williams *et al.* (1995), *supra*]. At least 90% of all reactivity, in either goat or chicken antibody pools, was found to be directed against the ligand binding domain (interval 6). The remaining immunoreactivity was directed against all five remaining

intervals, and was similar in both antibody pools, except that the chicken antibody showed a much lower reactivity against interval 2 than the goat antibody.

5 This clearly demonstrates that when native toxin A is used as an immunogen in goats or chickens, the bulk of the immune response is directed against the ligand binding domain of the protein, with the remaining response distributed throughout the remaining 2/3 of the protein.

**b) Affinity Purification Of IgY Reactive Against  
Recombinant Toxin A Protein**

10 Affinity columns, containing recombinant toxin A protein from the 6 defined intervals in (a) above, were made and used to (i) affinity purify antibodies reactive to each individual interval from the CTA IgY preparation [Example 8(c)], and (ii) deplete interval specific antibodies from the CTA IgY preparation. Affinity columns were made by coupling 1 ml of PBS-washed Actigel resin (Sterogene) with region specific protein and 1/10 final volume of Ald-coupling solution (1M sodium  
15 cyanoborohydride). The total region specific protein added to each reaction mixture was 2.7 mg (interval 1), 3 mg (intervals 2 and 3), 0.1 mg (interval 4), 0.2 mg (interval 5) and 4 mg (interval 6). Protein for intervals 1, 3, and 6 was affinity purified pMAL fusion protein in column buffer (see Example 11). Interval 4 was affinity purified poly-histidine containing pET fusion in PBS; intervals 2 and 5 were from inclusion  
20 body preparations of insoluble pMAL fusion protein, dialyzed extensively in PBS. Aliquots of the supernatants from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, in all cases greater than 50% coupling efficiencies were estimated. The resins were poured into 5 ml BioRad columns, washed extensively with PBS, and  
25 stored at 4°C.

Aliquots of the CTA IgY polyclonal antibody preparation were depleted for each individual region as described below. A 20 ml sample of the CTA IgY preparation [Example 8(c)] was dialyzed extensively against 3 changes of PBS (1 liter for each dialysis), quantitated by absorbance at OD<sub>280</sub>, and stored at 4°C. Six 1 ml

5 aliquots of the dialyzed IgY preparation were removed, and depleted individually for each of the six intervals. Each 1 ml aliquot was passed over the appropriate affinity column, and the eluate twice reappplied to the column. The eluate was collected, and pooled with a 1 ml PBS wash. Bound antibody was eluted from the column by  
10 washing with 5 column volumes of 4 M Guanidine-HCl (in 10 mM Tris-HCl, pH 8.0). The column was reequilibrated in PBS, and the depleted antibody stock reappplied as described above. The eluate was collected, pooled with a 1 ml PBS wash, quantitated by absorbance at OD<sub>280</sub>, and stored at 4° C. In this manner, 6 aliquots of the CTA IgY preparation were individually depleted for each of the 6 toxin A intervals, by two  
15 rounds of affinity depletion. The specificity of each depleted stock was tested by Western blot analysis. Multiple 7.5% SDS-PAGE gels were loaded with protein samples corresponding to all 6 toxin A subregions. After electrophoresis, the gels were blotted, and protein transfer confirmed by Ponceau S staining [protocols described in Williams *et al.* (1995), *supra*]. After blocking the blots 1 hr at 20°C in  
20 PBS+ 0.1% Tween 20 (PBST) containing 5% milk (as a blocking buffer), 4 ml of either a 1/500 dilution of the dialyzed CTA IgY preparation in blocking buffer, or an equivalent amount of the six depleted antibody stocks (using OD<sub>280</sub> to standardize antibody concentration) were added and the blots incubated a further 1 hr at room temperature. The blots were washed and developed with alkaline phosphatase (using a  
25 rabbit anti-chicken alkaline phosphate conjugate as a secondary antibody) as previously described [Williams *et al.* (1995), *supra*]. In all cases, only the target interval was depleted for antibody reactivity, and at least 90% of the reactivity to the target intervals was specifically depleted.

25 Region specific antibody pools were isolated by affinity chromatography as described below. Ten mls of the dialyzed CTA IgY preparation were applied sequentially to each affinity column, such that a single 10 ml aliquot was used to isolate region specific antibodies specific to each of the six subregions. The columns were sequentially washed with 10 volumes of PBS, 6 volumes of BBS-Tween, 10 volumes of TBS, and eluted with 4 ml Actisep elution media (Sterogene). The eluate  
30 was dialyzed extensively against several changes of PBS, and the affinity purified

antibody collected and stored at 4°C. The volumes of the eluate increased to greater than 10 mls during dialysis in each case, due to the high viscosity of the Actisep elution media. Aliquots of each sample were 20x concentrated using Centricon 30 microconcentrators (Amicon) and stored at 4°C. The specificity of each region specific antibody pool was tested, relative to the dialyzed CTA IgY preparation, by Western blot analysis, exactly as described above, except that 4 ml samples of blocking buffer containing 100 µl region specific antibody (unconcentrated) were used instead of the depleted CTA IgY preparations. Each affinity purified antibody preparation was specific to the defined interval, except that samples purified against intervals 1-5 also reacted with interval 6. This may be due to non-specific binding to the interval 6 protein, since this protein contains the repetitive ligand binding domain which has been shown to bind antibodies nonspecifically. [Lyerly *et al.*, Curr. Microbiol., 19:303-306 (1989).]

The reactivity of each affinity purified antibody preparation to the corresponding proteins was approximately the same as the reactivity of the 1/500 diluted dialyzed CTA IgY preparation standard. Given that the specific antibody stocks were diluted 1/40, this would indicate that the unconcentrated affinity purified antibody stocks contain 1/10-1/20 the concentration of specific antibodies relative to the starting CTA IgY preparation.

**c) Toxin A Neutralization Assay Using Antibodies  
Reactive Toward Recombinant Toxin A Protein**

The CHO toxin neutralization assay [Example 8(d)] was used to assess the ability of the depleted or enriched samples generated in (b) above to neutralize the cytotoxicity of toxin A. The general ability of affinity purified antibodies to neutralize toxin A was assessed by mixing together aliquots of all 6 concentrated stocks of the 6 affinity purified samples generated in (b) above, and testing the ability of this mixture to neutralize a toxin A concentration of 0.1 µg/ml. The results, shown in Figure 11, demonstrate almost complete neutralization of toxin A using the affinity purified (AP) mix. Some epitopes within the recombinant proteins utilized for affinity purification



were probably lost when the proteins were denatured before affinity purification [by Guanidine-HCl treatment in (b) above]. Thus, the neutralization ability of antibodies directed against recombinant protein is probably underestimated using these affinity purified antibody pools. This experiment demonstrates that antibodies reactive to recombinant toxin A can neutralize cytotoxicity, suggesting that neutralizing antibodies may be generated by using recombinant toxin A protein as immunogen.

In view of the observation that the recombinant expression clones of the toxin A gene divide the protein into 6 subregions, the neutralizing ability of antibodies directed against each individual region was assessed. The neutralizing ability of antibodies directed against the ligand binding domain of toxin A was determined first.

In the toxin neutralization experiment shown in Figure 11, interval 6 specific antibodies (interval 6 contains the ligand binding domain) were depleted from the dialyzed PEG preparation, and the effect on toxin neutralization assayed. Interval 6 antibodies were depleted either by utilizing the interval 6 depleted CTA IgY preparation from (b) above ("-6 aff. depleted" in Figure 11), or by addition of interval 6 protein to the CTA IgY preparation (estimated to be a 10 fold molar excess over anti-interval 6 immunoglobulin present in this preparation) to competitively compete for interval 6 protein ("-6 prot depleted" in Figure 11). In both instances, removal of interval 6 specific antibodies reduces the neutralization efficiency relative to the starting CTA IgY preparation. This demonstrates that antibodies directed against interval 6 contribute to toxin neutralization. Since interval 6 corresponds to the ligand binding domain of the protein, these results demonstrate that antibodies directed against this region in the PEG preparation contribute to the neutralization of toxin A in this assay. However, it is significant that after removal of these antibodies, the PEG preparation retains significant ability to neutralize toxin A (Figure 11). This neutralization is probably due to the action of antibodies specific to other regions of the toxin A protein, since at least 90% of the ligand binding region reactive antibodies were removed in the depleted sample prepared in (b) above. This conclusion was supported by comparison of the toxin neutralization of the affinity purified (AP) mix compared to affinity purified interval 6 antibody alone. Although some neutralization

ability was observed with AP interval 6 antibodies alone, the neutralization was significantly less than that observed with the mixture of all 6 AP antibody stocks (not shown).

5        Given that the mix of all six affinity purified samples almost completely  
neutralized the cytotoxicity of toxin A (Figure 11), the relative importance of  
antibodies directed against toxin A intervals 1-5 within the mixture was determined.  
This was assessed in two ways. First, samples containing affinity purified antibodies  
representing 5 of the 6 intervals were prepared, such that each individual region was  
depleted from one sample. Figure 12 demonstrates a sample neutralization curve,  
10        comparing the neutralization ability of affinity purified antibody mixes without  
interval 4 (-4) or 5 (-5) specific antibodies, relative to the mix of all 6 affinity purified  
antibody stocks (positive control). While the removal of interval 5 specific antibodies  
had no effect on toxin neutralization (or intervals 1-3, not shown), the loss of  
interval 4 specific antibodies significantly reduced toxin neutralization (Figure 12).

15        Similar results were seen in a second experiment, in which affinity purified  
antibodies, directed against a single region, were added to interval 6 specific  
antibodies, and the effects on toxin neutralization assessed. Only interval 4 specific  
antibodies significantly enhanced neutralization when added to interval 6 specific  
antibodies (Figure 13). These results demonstrate that antibodies directed against  
20        interval 4 (corresponding to clone pPA1100-1450 in Figure 9) are important for  
neutralization of cytotoxicity in this assay. Epitope mapping has shown that only low  
levels of antibodies reactive to this region are generated when native toxin A is used  
as an immunogen [Example 12(a)]. It is hypothesized that immunization with  
recombinant protein specific to this interval will elicit higher titers of neutralizing  
25        antibodies. In summary, this analysis has identified two critical regions of the toxin A  
protein against which neutralizing antibodies are produced, as assayed by the CHO  
neutralization assay.

### EXAMPLE 13

#### Production And Evaluation Of Avian Antitoxin Against *C. difficile* Recombinant Toxin A Polypeptide

In Example 12, we demonstrated neutralization of toxin A mediated  
5 cytotoxicity by affinity purified antibodies reactive to recombinant toxin A protein. To  
determine whether antibodies raised against a recombinant polypeptide fragment of  
*C. difficile* toxin A may be effective in treating clostridial diseases, antibodies to  
recombinant toxin A protein representing the binding domain were generated. Two  
toxin A binding domain recombinant polypeptides, expressing the binding domain in  
10 either the pMALc (pMA1870-2680) or pET 23(pPA1870-2680) vector, were used as  
immunogens. The pMAL protein was affinity purified as a soluble product  
[Example 12(d)] and the pET protein was isolated as insoluble inclusion bodies  
[Example 12(d)] and solubilized to an immunologically active protein using a  
proprietary method described in a pending patent application (U.S. Patent Application  
15 Serial No. 08/129,027). This Example involves (a) immunization, (b) antitoxin  
collection, (c) determination of antitoxin antibody titer, (d) anti-recombinant toxin A  
neutralization of toxin A hemagglutination activity *in vitro*, and (e) assay of *in vitro*  
toxin A neutralizing activity.

#### a) Immunization

20 The soluble and the inclusion body preparations each were used separately to  
immunize hens. Both purified toxin A polypeptides were diluted in PBS and  
emulsified with approximately equal volumes of CFA for the initial immunization or  
IFA for subsequent booster immunizations. On day zero, for each of the recombinant  
preparations, two egg laying white Leghorn hens (obtained from local breeder) were  
25 each injected at multiple sites (intramuscular and subcutaneous) with 1 ml of  
recombinant adjuvant mixture containing approximately 0.5 to 1.5 mgs of recombinant  
toxin A. Booster immunizations of 1.0 mg were given on days 14 and day 28.

#### b) Antitoxin Collection

Total yolk immune IgY was extracted as described in the standard PEG protocol (as in Example 1) and the final IgY pellet was dissolved in sterile PBS at the original yolk volume. This material is designated "immune recombinant IgY" or "immune IgY."

#### c) Antitoxin Antibody Titer

To determine if the recombinant toxin A protein was sufficiently immunogenic to raise antibodies in hens, the antibody titer of a recombinant toxin A polypeptide was determined by ELISA. Eggs from both hens were collected on day 32, the yolks pooled and the antibody was isolated using PEG as described. The immune recombinant IgY antibody titer was determined for the soluble recombinant protein containing the maltose binding protein fusion generated in p-Mal (pMA1870-2680). Ninety-six well Falcon Pro-bind plates were coated overnight at 4°C with 100 µl /well of toxin A recombinant at 2.5 µg /µl in PBS containing 0.05% thimerosal. Another plate was also coated with maltose binding protein (MBP) at the same concentration, to permit comparison of antibody reactivity to the fusion partner. The next day, the wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 hour at 37°C. IgY isolated from immune or preimmune eggs was diluted in antibody diluent (PBS containing 1% BSA and 0.05% Tween-20), and added to the blocked wells and incubated for 1 hour at 37°C. The plates were washed three times with PBS with 0.05% Tween-20, then three times with PBS. Alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma) diluted 1:1000 in antibody diluent was added to the plate, and incubated for 1 hour at 37°C. The plates were washed as before and substrate was added, [p-nitrophenyl phosphate (Sigma)] at 1 mg/ml in 0.05M Na<sub>2</sub>CO<sub>3</sub>, pH 9.5 and 10 mM MgCl<sub>2</sub>. The plates were evaluated quantitatively on a Dynatech MR 300 Micro EPA plate reader at 410 nm about 10 minutes after the addition of substrate.

Based on these ELISA results, high antibody titers were raised in chickens immunized with the toxin A recombinant polypeptide. The recombinant appeared to

be highly immunogenic, as it was able to generate high antibody titers relatively quickly with few immunizations. Immune IgY titer directed specifically to the toxin A portion of the recombinant was higher than the immune IgY titer to its fusion partner, the maltose binding protein, and significantly higher than the preimmune IgY. ELISA titers (reciprocal of the highest dilution of IgY generating a signal) in the preimmune IgY to the MBP or the recombinant was <1:30 while the immune IgY titers to MBP and the toxin A recombinant were 1:18750 and > 1:93750 respectively. Importantly, the anti-recombinant antibody titers generated in the hens against the recombinant polypeptide is much higher, compared to antibodies to that region raised using native toxin A. The recombinant antibody titer to region 1870-2680 in the CTA antibody preparation is at least five-fold lower compared to the recombinant generated antibodies (1:18750 versus >1:93750). Thus, it appears a better immune response can be generated against a specific recombinant using that recombinant as the immunogen compared to the native toxin A.

This observation is significant, as it shows that because recombinant portions stimulate the production of antibodies, it is not necessary to use native toxin molecules to produce antitoxin preparations. Thus, the problems associated with the toxicity of the native toxin are avoided and large-scale antitoxin production is facilitated.

#### **d) Anti-Recombinant Toxin A Neutralization Of Toxin A Hemagglutination Activity *In Vitro***

Toxin A has hemagglutinating activity besides cytotoxic and enterotoxin properties. Specifically, toxin A agglutinates rabbit erythrocytes by binding to a trisaccharide (gal 1-3B1-4GlcNAc) on the cell surface. [H. Krivan *et al.*, Infect. Immun., 53:573-581 (1986).] We examined whether the anti-recombinant toxin A (immune IgY, antibodies raised against the insoluble product expressed in pET) can neutralize the hemagglutination activity of toxin A in vitro. The hemagglutination assay procedure used was described by H.C. Krivan *et al.* Polyethylene glycol-fractionated immune or preimmune IgY were pre-absorbed with citrated rabbit erythrocytes prior to performing the hemagglutination assay because we have found

that IgY alone can agglutinate red blood cells. Citrated rabbit red blood cells (RRBC's)(Cocalico) were washed twice by centrifugation at 450 x g with isotonic buffer (0.1 M Tris-HCl, 0.05 M NaCl, pH 7.2). RRBC-reactive antibodies in the IgY were removed by preparing a 10% RRBC suspension (made by adding packed cells to immune or preimmune IgY) and incubating the mixture for 1 hour at 37°C. The RRBCs were then removed by centrifugation. Neutralization of the hemagglutination activity of toxin A by antibody was tested in round-bottomed 96-well microtiter plates. Twenty-five µl of toxin A (36 µg /ml) (Tech Lab) in isotonic buffer was mixed with an equal volume of different dilutions of immune or preimmune IgY in isotonic buffer, and incubated for 15 minutes at room temperature. Then, 50 µl of a 1% RRBC suspension in isotonic buffer was added and the mixture was incubated for 3 hours at 4°C. Positive control wells containing the final concentration of 9 µg/ml of toxin A after dilution without IgY were also included. Hemagglutination activity was assessed visually, with a diffuse matrix of RRBC's coating the bottom of the well representing a positive hemagglutination reaction and a tight button of RRBC's at the bottom of the well representing a negative reaction. The anti-recombinant immune IgY neutralized toxin A hemagglutination activity, giving a neutralization titer of 1:8. However, preimmune IgY was unable to neutralize the hemagglutination ability of toxin A.

**e) Assay Of *In Vitro* Toxin A Neutralizing Activity**

The ability of the anti-recombinant toxin A IgY (immune IgY antibodies raised against pMA1870-2680, the soluble recombinant binding domain protein expressed in pMAL, designated as Anti-tox. A-2 in Figure 14 , and referred to as recombinant region 6) and pre-immune IgY, prepared as described in Example 8(c) above, to neutralize the cytotoxic activity of toxin A was assessed *in vitro* using the CHO cell cytotoxicity assay, and toxin A (Tech Lab) at a concentration of 0.1µg/ml, as described in Example 8(d) above. As additional controls, the anti-native toxin A IgY (CTA) and pre-immune IgY preparations described in Example 8(c) above were also tested. The results are shown in Figure 14.

The anti-recombinant toxin A IgY demonstrated only partial neutralization of the cytotoxic activity of toxin A, while the pre-immune IgY did not demonstrate any significant neutralizing activity.

#### EXAMPLE 14

5

##### *In vivo* Neutralization Of *C. difficile* Toxin A

The ability of avian antibodies (IgY) raised against recombinant toxin A binding domain to neutralize the enterotoxin activity of *C. difficile* toxin A was evaluated *in vivo* using Golden Syrian hamsters. The Example involved:

- 10 (a) preparation of the avian anti-recombinant toxin A IgY for oral administration;  
(b) *in vivo* protection of hamsters from *C. difficile* toxin A enterotoxicity by treatment of toxin A with avian anti-recombinant toxin A IgY; and (c) histologic evaluation of hamster ceca.

##### a) Preparation Of The Avian Anti-Recombinant Toxin A IgY For Oral Administration

15

Eggs were collected from hens which had been immunized with the recombinant *C. difficile* toxin A fragment pMA1870-2680 (described in Example 13, above). A second group of eggs purchased at a local supermarket was used as a pre-immune (negative) control. Egg yolk immunoglobulin (IgY) was extracted by PEG from the two groups of eggs as described in Example 8(c), and the final IgY pellets  
20 were solubilized in one-fourth the original yolk volume using 0.1M carbonate buffer (mixture of  $\text{NaHCO}_3$  and  $\text{Na}_2\text{CO}_3$ ), pH 9.5. The basic carbonate buffer was used in order to protect the toxin A from the acidic pH of the stomach environment.

**b)     *In vivo* Protection Of Hamsters Against *C. difficile*  
Toxin A Enterotoxicity By Treatment Of Toxin A  
With Avian Anti-recombinant Toxin A IgY**

5     In order to assess the ability of the avian anti-recombinant toxin A IgY,  
prepared in section (a) above to neutralize the *in vivo* enterotoxin activity of toxin A,  
an *in vivo* toxin neutralization model was developed using Golden Syrian hamsters.  
This model was based on published values for the minimum amount of toxin A  
required to elicit diarrhea (0.08 mg toxin A/Kg body wt.) and death (0.16 mg toxin  
A/Kg body wt.) in hamsters when administered orally (Lyerly *et al.* Infect. Immun.,  
10     47:349-352 (1985).

For the study, four separate experimental groups were used, with each group  
consisting of 7 female Golden Syrian hamsters (Charles River), approx. three and one-  
half weeks old, weighing approx. 50 gms each. The animals were housed as groups  
of 3 and 4, and were offered food and water *ad libitum* through the entire length of  
15     the study.

For each animal, a mixture containing either 10 $\mu$ g of toxin A (0.2 mg/Kg) or  
30 $\mu$ g of toxin A (0.6 mg/Kg) (*C. difficile* toxin A was obtained from Tech Lab and  
1 ml of either the anti-recombinant toxin A IgY or pre-immune IgY (from section (a)  
above) was prepared. These mixtures were incubated at 37°C for 60 min. and were  
20     then administered to the animals by the oral route. The animals were then observed  
for the onset of diarrhea and death for a period of 24 hrs. following the administration  
of the toxin A+IgY mixtures, at the end of which time, the following results were  
tabulated and shown in Table 17:



TABLE 17

Study Outcome At 24 Hours

Experimental Group	Study Outcome at 24 Hours		
	Healthy <sup>1</sup>	Diarrhea <sup>2</sup>	Dead <sup>3</sup>
10 µg Toxin A + Antitoxin Against Interval 6	7	0	0
30 µg Toxin A + Antitoxin Against Interval 6	7	0	0
10 µg Toxin A + Pre-Immune Serum	0	5	2
30 µg Toxin A + Pre-Immune	0	5	2

<sup>1</sup> Animals remained healthy through the entire 24 hour study period.

<sup>2</sup> Animals developed diarrhea, but did not die.

<sup>3</sup> Animals developed diarrhea, and subsequently died.

Pretreatment of toxin A at both doses tested, using the anti-recombinant toxin A IgY, prevented all overt symptoms of disease in hamsters. Therefore, pretreatment of *C. difficile* toxin A, using the anti-recombinant toxin A IgY, neutralized the *in vivo* enterotoxin activity of the toxin A. In contrast, all animals from the two groups which received toxin A which had been pretreated using pre-immune IgY developed disease symptoms which ranged from diarrhea to death. The diarrhea which developed in the 5 animals which did not die in each of the two pre-immune groups, spontaneously resolved by the end of the 24 hr. study period.

### c) Histologic Evaluation Of Hamster Ceca

In order to further assess the ability of anti-recombinant toxin A IgY to protect hamsters from the enterotoxin activity of toxin A, histologic evaluations were performed on the ceca of hamsters from the study described in section (b) above.

Three groups of animals were sacrificed in order to prepare histological specimens. The first group consisted of a single representative animal taken from each of the 4 groups of surviving hamsters at the conclusion of the study described in section (b) above. These animals represented the 24 hr. timepoint of the study.

The second group consisted of two animals which were not part of the study described above, but were separately treated with the same toxin A + pre-immune IgY

mixtures as described for the animals in section (b) above. Both of these hamsters developed diarrhea, and were sacrificed 8 hrs. after the time of administration of the toxin A + pre-immune IgY mixtures. At the time of sacrifice, both animals were presenting symptoms of diarrhea. These animals represented the acute phase of the study.

The final group consisted of a single untreated hamster from the same shipment of animals as those used for the two previous groups. This animal served as the normal control.

Samples of cecal tissue were removed from the 7 animals described above, and were fixed overnight at 4°C using 10% buffered formalin. The fixed tissues were paraffin-embedded, sectioned, and mounted on glass microscope slides. The tissue sections were then stained using hematoxylin and eosin (H and E stain), and were examined by light microscopy.

The tissues obtained from the two 24 hr. animals which received mixtures containing either 10µg or 30µg of toxin A and anti-recombinant toxin A IgY were indistinguishable from the normal control, both in terms of gross pathology, as well as at the microscopic level. These observations provide further evidence for the ability of anti-recombinant toxin A IgY to effectively neutralize the *in vivo* enterotoxin activity of *C. difficile* toxin A, and thus its ability to prevent acute or lasting toxin A-induced pathology.

In contrast, the tissues from the two 24 hr. animals which received the toxin A + pre-immune IgY mixtures demonstrated significant pathology. In both of these groups, the mucosal layer was observed to be less organized than in the normal control tissue. The cytoplasm of the epithelial cells had a vacuolated appearance, and gaps were present between the epithelium and the underlying cell layers. The lamina propria was largely absent. Intestinal villi and crypts were significantly diminished, and appeared to have been overgrown by a planar layer of epithelial cells and fibroblasts. Therefore, although these animals overtly appeared to recover from the acute symptoms of toxin A intoxication, lasting pathologic alterations to the cecal mucosa had occurred.

The tissues obtained from the two acute animals which received mixtures of toxin A and pre-immune IgY demonstrated the most significant pathology. At the gross pathological level, both animals were observed to have severely distended ceca which were filled with watery, diarrhea-like material. At the microscopic level, the animal that was given the mixture containing 10µg of toxin A and pre-immune IgY was found to have a mucosal layer which had a ragged, damaged appearance, and a disorganized, compacted quality. The crypts were largely absent, and numerous breaks in the epithelium had occurred. There was also an influx of erythrocytes into spaces between the epithelial layer and the underlying tissue. The animal which had received the mixture containing 30µg of toxin A and pre-immune IgY demonstrated the most severe pathology. The cecal tissue of this animal had an appearance very similar to that observed in animals which had died from *C. difficile* disease. Widespread destruction of the mucosa was noted, and the epithelial layer had sloughed. Hemorrhagic areas containing large numbers of erythrocytes were very prevalent. All semblance of normal tissue architecture was absent from this specimen. In terms of the presentation of pathologic events, this *in vivo* hamster model of toxin A-intoxication correlates very closely with the pathologic consequences of *C. difficile* disease in hamsters. The results presented in this Example demonstrate that while anti-recombinant toxin A (Interval 6) IgY is capable of only partially neutralizing the cytotoxic activity of *C. difficile* toxin A, the same antibody effectively neutralizes 100% of the *in vivo* enterotoxin activity of the toxin. While it is not intended that this invention be limited to this mechanism, this may be due to the cytotoxicity and enterotoxicity of *C. difficile* Toxin A as two separate and distinct biological functions.

## EXAMPLE 15

### *In Vivo* Neutralization Of *C. Difficile* Toxin A By Antibodies Against Recombinant Toxin A Polypeptides

5 The ability of avian antibodies directed against the recombinant *C. difficile*  
toxin A fragment 1870-2680 (as expressed by pMA1870-2680; see Example 13) to  
neutralize the enterotoxic activity of toxin A was demonstrated in Example 14. The  
ability of avian antibodies (IgYs) directed against other recombinant toxin A epitopes  
to neutralize native toxin A *in vivo* was next evaluated. This example involved: (a)  
the preparation of IgYs against recombinant toxin A polypeptides; (b) *in vivo*  
10 protection of hamsters against toxin A by treatment with anti-recombinant toxin A  
IgYs and (c) quantification of specific antibody concentration in CTA and Interval 6  
IgY PEG preparations.

The nucleotide sequence of the coding region of the entire toxin A protein is  
listed in SEQ ID NO:5. The amino acid sequence of the entire toxin A protein is  
15 listed in SEQ ID NO:6. The amino acid sequence consisting of amino acid residues  
1870 through 2680 of toxin A is listed in SEQ ID NO:7. The amino acid sequence  
consisting of amino acid residues 1870 through 1960 of toxin A is listed in SEQ ID  
NO:8.

#### a) Preparation Of IgY's Against Recombinant Toxin A 20 Polypeptides

Eggs were collected from Leghorn hens which have been immunized with  
recombinant *C. difficile* toxin A polypeptide fragments encompassing the entire toxin  
A protein. The polypeptide fragments used as immunogens were: 1) pMA 1870-2680  
(Interval 6), 2) pPA 1100-1450 (Interval 4), and 3) a mixture of fragments consisting  
25 of pMA 30-300 (Interval 1), pMA 300-660 (Interval 2), pMA 660-1100 (Interval 3)  
and pMA 1450-1870 (Interval 5). This mixture of immunogens is referred to as  
Interval 1235. The location of each interval within the toxin A molecule is shown in

Figure 15A. In Figure 15A, the following abbreviations are used: pP refers to the pET23 vector (New England BioLabs); pM refers to the pMAL<sup>TM</sup>-c vector (New England BioLabs); A refers to toxin A; the numbers refer to the amino acid interval expressed in the clone. (For example, the designation pMA30-300 indicates that the recombinant clone encodes amino acids 30-300 of toxin A and the vector used was pMAL<sup>TM</sup>-c).

The recombinant proteins were generated as described in Example 11. The IgYs were extracted and solubilized in 0.1M carbonate buffer pH 9.5 for oral administration as described in Example 14(a). The IgY reactivities against each individual recombinant interval was evaluated by ELISA as described in Example 13(c).

**b)     *In Vivo* Protection Of Hamsters Against Toxin  
          A By Treatment With Anti-Recombinant Toxin  
          A Antibodies**

The ability of antibodies raised against recombinant toxin A polypeptides to provide *in vivo* protection against the enterotoxic activity of toxin A was examined in the hamster model system. This assay was performed as described in Example 14(b). Briefly, for each 40-50 gram female Golden Syrian hamster (Charles River), 1 ml of IgY 4X (*i.e.*, resuspended in 1/4 of the original yolk volume) PEG prep against Interval 6, Interval 4 or Interval 1235 was mixed with 30 µg (LD<sub>100</sub> oral dose) of *C. difficile* toxin A (Tech Lab). Preimmune IgY mixed with toxin A served as a negative control. Antibodies raised against *C. difficile* toxoid A (Example 8) mixed with toxin A (CTA) served as a positive control. The mixture was incubated for 1 hour at 37°C then orally administered to lightly etherized hamsters using an 18G feeding needle. The animals were then observed for the onset of diarrhea and death for a period of approximately 24 hours. The results are shown in Table 18.

TABLE 18

Study Outcome After 24 Hours

Treatment group	Healthy <sup>1</sup>	Diarrhea <sup>2</sup>	Dead <sup>3</sup>
Preimmune	0	0	7
CTA	5	0	0
Interval 6	6	1	0
Interval 4	0	1	6
Interval 1235	0	0	7

<sup>1</sup> Animal shows no sign of illness.<sup>2</sup> Animal developed diarrhea, but did not die.<sup>3</sup> Animal developed diarrhea and died.

Pre-treatment of toxin A with IgYs against Interval 6 prevented diarrhea in 6 of 7 hamsters and completely prevented death in all 7. In contrast, as with preimmune IgY, IgYs against Interval 4 and Interval 1235 had no effect on the onset of diarrhea and death in the hamsters.

### c) Quantification Of Specific Antibody Concentration

#### In CTA And Interval 6 IgY PEG Preparations

To determine the purity of IgY PEG preparations, an aliquot of a pMA1870-2680 (Interval 6) IgY PEG preparation was chromatographed using HPLC and a KW-803 sizing column (Shodex). The resulting profile of absorbance at 280 nm is shown in Figure 16. The single large peak corresponds to the predicted MW of IgY. Integration of the area under the single large peak showed that greater than 95% of the protein eluted from the column was present in this single peak. This result demonstrated that the majority (>95%) of the material absorbing at 280 nm in the PEG preparation corresponds to IgY. Therefore, absorbance at 280 nm can be used to determine the total antibody concentration in PEG preparations.

To determine the concentration of Interval 6-specific antibodies (expressed as percent of total antibody) within the CTA and pMA1870-2680 (Interval 6) PEG preparations, defined quantities of these antibody preparations were affinity purified on

a pPA1870-2680(H) (shown schematically in Figure 15B) affinity column and the specific antibodies were quantified. In Figure 15B the following abbreviations are used: pP refers to the pET23 vector (New England BioLabs); pM refers to the pMAL<sup>TM</sup>-c vector (New England BioLabs); pG refers to the pGEX vector (Pharmacia); pB refers to the PinPoint<sup>TM</sup> Xa vector (Promega); A refers to toxin A; the numbers refer to the amino acid interval expressed in the clone. The solid black ovals represent the MBP; the hatched ovals represent glutathione S-transferase; the hatched circles represent the biotin tag; and HHH represents the poly-histidine tag.

An affinity column containing recombinant toxin A repeat protein was made as follows. Four ml of PBS-washed Actigel resin (Sterogene) was coupled with 5-10 mg of pPA1870-2680 inclusion body protein [prepared as described in Example (17) and dialyzed into PBS] in a 15 ml tube (Falcon) containing 1/10 final volume Ald-coupling solution (1 M sodium cyanoborohydride). Aliquots of the supernatant from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based upon protein band intensities, greater than 6 mg of recombinant protein was coupled to the resin. The resin was poured into a 10 ml column (BioRad), washed extensively with PBS, pre-eluted with 4 M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal) and re-equilibrated with PBS. The column was stored at 4°C.

Aliquots of a pMA1870-2680 (Interval 6) or a CTA IgY polyclonal antibody preparation (PEG prep) were affinity purified on the above affinity column as follows. The column was attached to an UV monitor (ISCO) and washed with PBS. For pMA1870-2680 IgY purification, a 2X PEG prep (filter sterilized using a 0.45  $\mu$  filter; approximately 500 mg total IgY) was applied. The column was washed with PBS until the baseline was re-established (the column flow-through was saved), washed with BBSTween to elute nonspecifically binding antibodies and re-equilibrated with PBS. Bound antibody was eluted from the column in 4 M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal). The entire elution peak was collected in a 15 ml tube (Falcon). The column was re-equilibrated and the column eluate was re-chromatographed as described above. The antibody preparation was quantified by UV

absorbance (the elution buffer was used to zero the spectrophotometer). Total purified antibody was approximately 9 mg and 1 mg from the first and second chromatography passes, respectively. The low yield from the second pass indicated that most specific antibodies were removed by the first round of chromatography. The estimated percentage of Interval 6 specific antibodies in the pMA1870-2680 PEG prep is approximately 2%.

The percentage of Interval 6 specific antibodies in the CTA PEG prep was determined (utilizing the same column and methodology described above) to be approximately 0.5% of total IgY.

A 4X PEG prep contains approximately 20 mg/ml IgY. Thus in b) above, approximately 400 µg specific antibody in the Interval 6 PEG prep neutralized 30 µg toxin A *in vivo*.

#### EXAMPLE 16

##### *In Vivo* Treatment Of *C. difficile* Disease In Hamsters By Recombinant Interval 6 Antibodies

The ability of antibodies directed against recombinant Interval 6 of toxin A to protect hamsters *in vivo* from *C. difficile* disease was examined. This example involved: (a) prophylactic treatment of *C. difficile* disease and (b) therapeutic treatment of *C. difficile* disease.

##### a) Prophylactic Treatment Of *C. difficile* Disease

This experiment was performed as described in Example 9(b). Three groups each consisting of 7 female 100 gram Syrian hamsters (Charles River) were prophylactically treated with either preimmune IgYs, IgYs against native toxin A and B [CTAB; see Example 8 (a) and (b)] or IgYs against Interval 6. IgYs were prepared as 4X PEG preparations as described in Example 9(a).



The animals were orally dosed 3 times daily, roughly at 4 hour intervals, for 12 days with 1 ml antibody preparations diluted in Ensure®. Using estimates of specific antibody concentration from Example 15(c), each dose of the Interval 6 antibody prep contained approximately 400 µg of specific antibody. On day 2 each hamster was predisposed to *C. difficile* infection by the oral administration of 3.0 mg of Clindamycin-HCl (Sigma) in 1 ml of water. On day 3 the hamsters were orally challenged with 1 ml of *C. difficile* inoculum strain ATCC 43596 in sterile saline containing approximately 100 organisms. The animals were then observed for the onset of diarrhea and subsequent death during the treatment period. The results are shown in Table 19.

TABLE 19

Lethality After 12 Days Of Treatment

Treatment Group	Number Animals Alive	Number Animals Dead
Preimmune	0	7
CTAB	6	1
Interval 6	7	0

Treatment of hamsters with orally-administered IgYs against Interval 6 successfully protected 7 out of 7 (100%) of the animals from *C. difficile* disease. One of the hamsters in this group presented with diarrhea which subsequently resolved during the course of treatment. As shown previously in Example 9, antibodies to native toxin A and toxin B were highly protective. In this Example, 6 out of 7 animals survived in the CTAB treatment group. All of the hamsters treated with preimmune sera came down with diarrhea and died. The survivors in both the CTAB and Interval 6 groups remained healthy throughout a 12 day post-treatment period. In particular, 6 out of 7 Interval 6-treated hamsters survived at least 2 weeks after termination of treatment which suggests that these antibodies provide a long-lasting cure. These results represent the first demonstration that antibodies generated against a recombinant region of toxin A can prevent CDAD when administered passively to animals. These results also indicate that antibodies raised against Interval 6 alone may

be sufficient to protect animals from *C. difficile* disease when administered prophylactically.

Previously others had raised antibodies against toxin A by actively immunizing hamsters against a recombinant polypeptide located within the Interval 6 region [Lyerly, D.M., *et al.* (1990) *Curr. Microbiol.* 21:29]. Figure 17 shows schematically the location of the Lyerly, *et al.* intra-Interval 6 recombinant protein (cloned into the pUC vector) in comparison with the complete Interval 6 construct (pMA1870-2680) used herein to generate neutralizing antibodies directed against toxin A. In Figure 17, the solid black oval represents the MBP which is fused to the toxin A Interval 6 in pMA1870-2680.

The Lyerly, *et al.* antibodies (intra-Interval 6) were only able to partially protect hamsters against *C. difficile* infection in terms of survival (4 out of 8 animals survived) and furthermore, these antibodies did not prevent diarrhea in any of the animals. Additionally, animals treated with the intra-Interval 6 antibodies [Lyerly, *et al.* (1990), *supra*] died when treatment was removed.

In contrast, the experiment shown above demonstrates that passive administration of anti-Interval 6 antibodies prevented diarrhea in 6 out of 7 animals and completely prevented death due to CDAD. Furthermore, as discussed above, passive administration of the anti-Interval 6 antibodies provides a long lasting cure (*i.e.*, treatment could be withdrawn without incident).

**b) Therapeutic Treatment Of *C. difficile* Disease: *In Vivo*  
Treatment Of An Established *C. difficile* Infection In  
Hamsters With Recombinant Interval 6 Antibodies**

The ability of antibodies against recombinant interval 6 of toxin A to therapeutically treat *C. difficile* disease was examined. The experiment was performed essentially as described in Example 10(b). Three groups, each containing seven to eight female Golden Syrian hamsters (100 g each; Charles River) were treated with either preimmune IgY, IgYs against native toxin A and toxin B (CTAB) and IgYs

against Interval 6. The antibodies were prepared as described above as 4X PEG preparations.

5 The hamsters were first predisposed to *C. difficile* infection with a 3 mg dose of Clindamycin-HCl (Sigma) administered orally in 1 ml of water. Approximately 24 hrs later, the animals were orally challenged with 1 ml of *C. difficile* strain ATCC 43596 in sterile saline containing approximately 200 organisms. One day after infection, the presence of toxin A and B was determined in the feces of the hamsters using a commercial immunoassay kit (Cytoclone A+B EPA, Cambridge Biotech) to verify establishment of infection. Four members of each group were randomly selected and tested. Feces from an uninfected hamster was tested as a negative control. All infected animals tested positive for the presence of toxin according to the manufacturer's procedure. The initiation of treatment then started approximately 24 hr post-infection.

15 The animals were dosed daily at roughly 4 hr intervals with 1 ml antibody preparation diluted in Ensure® (Ross Labs). The amount of specific antibodies given per dose (determined by affinity purification) was estimated to be about 400 µg of anti-Interval 6 IgY (for animals in the Interval 6 group) and 100 µg and 70 µg of anti-toxin A (Interval 6-specific) and anti-toxin B (Interval 3-specific; see Example 19), respectively, for the CTAB preparation. The animals were treated for 9 days and then observed for an additional 4 days for the presence of diarrhea and death. The results indicating the number of survivors and the number of dead 4 days post-infection are shown in Table 20.

TABLE 20

*In vivo* Therapeutic Treatment With Interval 6 Antibodies

25

Treatment Group	Number Animals Alive	Number Animals Dead
Preimmune	4	3
CTAB	8	0
Interval 6	8	0

Antibodies directed against both Interval 6 and CTAB successfully prevented death from *C. difficile* when therapeutically administered 24 hr after infection. This result is significant since many investigators begin therapeutic treatment of hamsters with existing drugs (*e.g.*, vancomycin, phenelfamycins, tiacumicins, etc.) 8 hr post-infection [Swanson, *et al.* (1991) Antimicrobial Agents and Chemotherapy 35:1108 and (1989) J. Antibiotics 42:94].

Forty-two percent of hamsters treated with preimmune IgY died from CDAD. While the anti-Interval 6 antibodies prevented death in the treated hamsters, they did not eliminate all symptoms of CDAD as 3 animals presented with slight diarrhea. In addition, one CTAB-treated and one preimmune-treated animal also had diarrhea 14 days post-infection. These results indicate that anti-Interval 6 antibodies provide an effective means of therapy for CDAD.

#### EXAMPLE 17

Induction Of Toxin A Neutralizing Antibodies Requires Soluble Interval 6 Protein

As shown in Examples 11(d) and 15, expression of recombinant proteins in *E. coli* may result in the production of either soluble or insoluble protein. If insoluble protein is produced, the recombinant protein is solubilized prior to immunization of animals. To determine whether, one or both of the soluble or insoluble recombinant proteins could be used to generate neutralizing antibodies to toxin A, the following experiment was performed. This example involved a) expression of the toxin A repeats and subfragments of these repeats in *E. coli* using a variety of expression vectors; b) identification of recombinant toxin A repeats and sub-regions to which neutralizing antibodies bind; and c) determination of the neutralization ability of antibodies raised against soluble and insoluble toxin A repeat immunogen.

a) **Expression Of The Toxin A Repeats And  
Subfragments Of These Repeats In *E. coli*  
Using A Variety Of Expression Vectors**

5 The Interval 6 immunogen utilized in Examples 15 and 16 was the pMA1870-2680 protein, in which the toxin A repeats are expressed as a soluble fusion protein with the MBP (described in Example 11). Interestingly, expression of this region (from the *SpeI* site to the end of the repeats, see Figure 15B) in three other expression constructs, as either native (pPA1870-2680), poly-His tagged [pPA1870-2680 (H)] or biotin-tagged (pBA1870-2680) proteins resulted in completely insoluble protein upon  
10 induction of the bacterial host (see Figure 15B). The host strain BL21 (Novagen) was used for expression of pBA1870-2680 and host strain BL21(DE3) (Novagen) was used for expression of pPA1870-2680 and pPA1870-2680(H). These insoluble proteins accumulated to high levels in inclusion bodies. Expression of recombinant plasmids in *E. coli* host cells grown in 2X YT medium was performed as described [Williams, *et al.* (1995), *supra*].  
15

As summarized in Figure 15B, expression of fragments of the toxin A repeats (as either N-terminal *SpeI*-*EcoRI* fragments, or C-terminal *EcoRI*-end fragments) also yielded high levels of insoluble protein using pGEX (pGA1870-2190), PinPoint™-Xa (pBA1870-2190 and pBA2250-2680) and pET expression systems (pPA1870-2190).  
20 The pGEX and pET expression systems are described in Example 11. The PinPoint™-Xa expression system drives the expression of fusion proteins in *E. coli*. Fusion proteins from PinPoint™-Xa vectors contain a biotin tag at the amino-terminal end and can be affinity purified SoftLink™ Soft Release avidin resin (Promega) under mild denaturing conditions (5 mM biotin).

25 The solubility of expressed proteins from the pPG1870-2190 and pPA1870-2190 expression constructs was determined after induction of recombinant protein expression under conditions reported to enhance protein solubility [These conditions comprise growth of the host at reduced temperature (30°C) and the utilization of high (1 mM IPTG) or low (0.1 mM IPTG) concentrations of inducer [Williams *et al.*

(1995), *supra*]. All expressed recombinant toxin A protein was insoluble under these conditions. Thus, expression of these fragments of the toxin A repeats in pET and pGEX expression vectors results in the production of insoluble recombinant protein even when the host cells are grown at reduced temperature and using lower concentrations of the inducer. Although expression of these fragments in pMal vectors yielded affinity purifiable soluble fusion protein, the protein was either predominantly insoluble (pMA1870-2190) or unstable (pMA2250-2650). Attempts to solubilize expressed protein from the pMA1870-2190 expression construct using reduced temperature or lower inducer concentration (as described above) did not improve fusion protein solubility.

Collectively, these results demonstrate that expression of the toxin A repeat region in *E. coli* results in the production of insoluble recombinant protein, when expressed as either large (aa 1870-2680) or small (aa 1870-2190 or aa 2250-2680) fragments, in a variety of expression vectors (native or poly-his tagged pET, pGEX or PinPoint™- Xa vectors), utilizing growth conditions shown to enhance protein solubility. The exception to this rule were fusions with the MBP, which enhanced protein solubility, either partially (pMA1870-2190) or fully (pMA1870-2680).

#### **b) Identification Of Recombinant Toxin A Repeats And Sub-Regions To Which Neutralizing Antibodies Bind**

Toxin A repeat regions to which neutralizing antibodies bind were identified by utilizing recombinant toxin A repeat region proteins expressed as soluble or insoluble proteins to deplete protective antibodies from a polyclonal pool of antibodies against native *C. difficile* toxin A. An *in vivo* assay was developed to evaluate proteins for the ability to bind neutralizing antibodies.

The rationale for this assay is as follows. Recombinant proteins were first pre-mixed with antibodies against native toxin A (CTA antibody; generated in Example 8) and allowed to react. Subsequently, *C. difficile* toxin A was added at a concentration lethal to hamsters and the mixture was administered to hamsters via IP injection. If

the recombinant protein contains neutralizing epitopes, the CTA antibodies would lose their ability to bind toxin A resulting in diarrhea and/or death of the hamsters.

The assay was performed as follows. The lethal dose of toxin A when delivered orally to nine 40 to 50 g Golden Syrian hamsters (Sasco) was determined to be 10 to 30  $\mu$ g. The PEG-purified CTA antibody preparation was diluted to 0.5X concentration (*i.e.*, the antibodies were diluted at twice the original yolk volume) in 0.1 M carbonate buffer, pH 9.5. The antibodies were diluted in carbonate buffer to protect them from acid degradation in the stomach. The concentration of 0.5X was used because it was found to be the lowest effective concentration against toxin A. The concentration of Interval 6-specific antibodies in the 0.5X CTA prep was estimated to be 10-15  $\mu$ g/ml (estimated using the method described in Example 15).

The inclusion body preparation [insoluble Interval 6 protein; pPA1870-2680(H)] and the soluble Interval 6 protein [pMA1870-2680; see Figure 15] were both compared for their ability to bind to neutralizing antibodies against *C. difficile* toxin A (CTA). Specifically, 1 to 2 mg of recombinant protein was mixed with 5 ml of a 0.5X CTA antibody prep (estimated to contain 60-70  $\mu$ g of Interval 6-specific antibody). After incubation for 1 hr at 37°C, CTA (Tech Lab) at a final concentration of 30  $\mu$ g/ml was added and incubated for another 1 hr at 37°C. One ml of this mixture containing 30  $\mu$ g of toxin A (and 10-15  $\mu$ g of Interval 6-specific antibody) was administered orally to 40-50 g Golden Syrian hamsters (Sasco). Recombinant proteins that result in the loss of neutralizing capacity of the CTA antibody would indicate that those proteins contain neutralizing epitopes. Preimmune and CTA antibodies (both at 0.5X) without the addition of any recombinant protein served as negative and positive controls, respectively.

Two other inclusion body preparations, both expressed as insoluble products in the pET vector, were tested; one containing a different insert (toxin B fragment) other than Interval 6 called pPB1850-2070 (see Figure 18) which serves as a control for insoluble Interval 6, the other was a truncated version of the Interval 6 region called pPA1870-2190 (see Figure 15B). The results of this experiment are shown in Table 21.

TABLE 21

Binding Of Neutralizing Antibodies By Soluble Interval 6 Protein Study Outcome After 24 Hours

Treatment Group <sup>1</sup>	Healthy <sup>2</sup>	Diarrhea <sup>3</sup>	Dead <sup>4</sup>
Preimmune Ab	0	3	2
CTA Ab	4	1	0
CTA Ab + Int 6 (soluble)	1	2	2
CTA Ab + Int 6 (insoluble)	5	0	0
CTA Ab + pPB1850-2070	5	0	0
CTA Ab + pPA1870-2190	5	0	0

<sup>1</sup> *C. difficile* toxin A (CTA) was added to each group.<sup>2</sup> Animals showed no signs of illness.<sup>3</sup> Animals developed diarrhea but did not die.<sup>4</sup> Animals developed diarrhea and died.

Preimmune antibody was ineffective against toxin A, while anti-CTA antibodies at a dilute 0.5X concentration almost completely protected the hamsters against the enterotoxic effects of CTA. The addition of recombinant proteins pPB1850-2070 or pPA1870-2190 to the anti-CTA antibody had no effect upon its protective ability, indicating that these recombinant proteins do not bind to neutralizing antibodies. On the other hand, recombinant Interval 6 protein was able to bind to neutralizing anti-CTA antibodies and neutralized the *in vivo* protective effect of the anti-CTA antibodies. Four out of five animals in the group treated with anti-CTA antibodies mixed with soluble Interval 6 protein exhibited toxin associated toxicity (diarrhea and death). Moreover, the results showed that Interval 6 protein must be expressed as a soluble product in order for it to bind to neutralizing anti-CTA antibodies since the addition of insoluble Interval 6 protein had no effect on the neutralizing capacity of the CTA antibody prep.

**c) Determination Of Neutralization Ability Of Antibodies  
Raised Against Soluble And Insoluble Toxin A Repeat  
Immunogen**



To determine if neutralizing antibodies are induced against solubilized inclusion bodies, insoluble toxin A repeat protein was solubilized and specific antibodies were raised in chickens. Insoluble pPA1870-2680 protein was solubilized using the method described in Williams *et al.* (1995), *supra*. Briefly, induced cultures (500 ml) were pelleted by centrifugation at 3,000 X g for 10 min at 4°C. The cell pellets were resuspended thoroughly in 10 ml of inclusion body sonication buffer (25 mM HEPES pH 7.7, 100 mM KCl, 12.5 mM MgCl<sub>2</sub>, 20% glycerol, 0.1% (v/v) Nonidet P-40, 1 mM DTT). The suspension was transferred to a 30 ml non-glass centrifuge tube. Five hundred µl of 10 mg/ml lysozyme was added and the tubes were incubated on ice for 30 min. The suspension was then frozen at -70°C for at least 1 hr. The suspension was thawed rapidly in a water bath at room temperature and then placed on ice. The suspension was then sonicated using at least eight 15 sec bursts of the microprobe (Branson Sonicator Model No. 450) with intermittent cooling on ice.

The sonicated suspension was transferred to a 35 ml Oakridge tube and centrifuged at 6,000 X g for 10 min at 4°C to pellet the inclusion bodies. The pellet was washed 2 times by pipetting or vortexing in fresh, ice-cold RIPA buffer [0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate in TBS (25 mM Tris-Cl pH 7.5, 150 mM NaCl)]. The inclusion bodies were recentrifuged after each wash. The inclusion bodies were dried and transferred using a small metal spatula to a 15 ml tube (Falcon). One ml of 10% SDS was added and the pellet was solubilized by gently pipetting the solution up and down using a 1 ml micropipettor. The solubilization was facilitated by heating the sample to 95°C when necessary.

Once the inclusion bodies were in solution, the samples were diluted with 9 volumes of PBS. The protein solutions were dialyzed overnight against a 100-fold volume of PBS containing 0.05% SDS at room temperature. The dialysis buffer was then changed to PBS containing 0.01% SDS and the samples were dialyzed for several hours to overnight at room temperature. The samples were stored at 4°C until used. Prior to further use, the samples were warmed to room temperature to allow any precipitated SDS to go back into solution.

The inclusion body preparation was used to immunize hens. The protein was dialyzed into PBS and emulsified with approximately equal volumes of CFA for the initial immunization or IFA for subsequent booster immunizations. On day zero, for each of the recombinant recombinant preparations, two egg laying white Leghorn hens were each injected at multiple sites (IM and SC) with 1 ml of recombinant protein-adjuvant mixture containing approximately 0.5-1.5 mg of recombinant protein. Booster immunizations of 1.0 mg were given of days 14 and day 28. Eggs were collected on day 32 and the antibody isolated using PEG as described in Example 14(a). High titers of toxin A specific antibodies were present (as assayed by ELISA, using the method described in Example 13). Titers were determined for both antibodies against recombinant polypeptides pPA1870-2680 and pMA1870-2680 and were found to be comparable at > 1:62,500.

Antibodies against soluble Interval 6 (pMA1870-2680) and insoluble Interval 6 [(inclusion body), pPA1870-2680] were tested for neutralizing ability against toxin A using the *in vivo* assay described in Example 15(b). Preimmune antibodies and antibodies against toxin A (CTA) served as negative and positive controls, respectively. The results are shown in Table 22.

TABLE 22

Neutralization Of Toxin A By Antibodies Against  
Soluble Interval 6 Protein Study Outcome After 24 Hours

Antibody Treatment Group	Healthy <sup>1</sup>	Diarrhea <sup>2</sup>	Dead <sup>3</sup>
Preimmune	1	0	4
CTA	5	0	0
Interval 6 (Soluble) <sup>4</sup>	5	0	0
Interval 6 (Insoluble)	0	2	3

<sup>1</sup> Animals showed no sign of illness.

<sup>2</sup> Animal developed diarrhea but did not die.

<sup>3</sup> Animal developed diarrhea and died.

<sup>4</sup> 400 µg/ml.

Antibodies raised against native toxin A were protective while preimmune antibodies had little effect. As found using the *in vitro* CHO assay [described in

Example 8(d)] where antibodies raised against the soluble Interval 6 could partially neutralize the effects of toxin A, here they were able to completely neutralize toxin A *in vivo*. In contrast, the antibodies raised against the insoluble Interval 6 was unable to neutralize the effects of toxin A *in vivo* as shown above (Table 22) and *in vitro* as shown in the CHO assay [described in Example 8(d)].

These results demonstrate that soluble toxin A repeat immunogen is necessary to induce the production of neutralizing antibodies in chickens, and that the generation of such soluble immunogen is obtained only with a specific expression vector (pMal) containing the toxin A region spanning aa 1870-2680. That is to say, insoluble protein that is subsequently solubilized does not result in a toxin A antigen that will elicit a neutralizing antibody.

### EXAMPLE 18

#### Cloning And Expression Of The *C. difficile* Toxin B Gene

The toxin B gene has been cloned and sequenced; the amino acid sequence deduced from the cloned nucleotide sequence predicts a MW of 269.7 kD for toxin B [Barroso *et al.*, Nucl. Acids Res. 18:4004 (1990)]. The nucleotide sequence of the coding region of the entire toxin B gene is listed in SEQ ID NO:9. The amino acid sequence of the entire toxin B protein is listed in SEQ ID NO:10. The amino acid sequence consisting of amino acid residues 1850 through 2360 of toxin B is listed in SEQ ID NO:11. The amino acid sequence consisting of amino acid residues 1750 through 2360 of toxin B is listed in SEQ ID NO:12.

Given the expense and difficulty of isolating native toxin B protein, it would be advantageous to use simple and inexpensive procaryotic expression systems to produce and purify high levels of recombinant toxin B protein for immunization purposes. Ideally, the isolated recombinant protein would be soluble in order to preserve native antigenicity, since solubilized inclusion body proteins often do not fold into native conformations. Indeed as shown in Example 17, neutralizing antibodies against recombinant toxin A were only obtained when soluble recombinant toxin A

polypeptides were used as the immunogen. To allow ease of purification, the recombinant protein should be expressed to levels greater than 1 mg/liter of *E. coli* culture.

5 To determine whether high levels of recombinant toxin B protein could be produced in *E. coli*, fragments of the toxin B gene were cloned into various prokaryotic expression vectors, and assessed for the ability to express recombinant toxin B protein in *E. coli*. This Example involved (a) cloning of the toxin B gene and (b) expression of the toxin B gene in *E. coli*.

#### a) Cloning Of The Toxin B Gene

10 The toxin B gene was cloned using PCR amplification from *C. difficile* genomic DNA. Initially, the gene was cloned in two overlapping fragments, using primer pairs P5/P6 and P7/P8. The location of these primers along the toxin B gene is shown schematically in Figure 18. The sequence of each of these primers is:  
15 P5: 5' TAGAAAAAATGGCAAATGT 3' (SEQ ID NO:11); P6: 5' TTTCATCTTGTA GAGTCAAAG 3' (SEQ ID NO:12); P7: 5' GATGCCACAAGATGATTAGTG 3' (SEQ ID NO:13); and P8: 5' CTAATTGAGCTGTATCAGGATC 3' (SEQ ID NO:14).

Figure 18 also shows the location of the following primers along the toxin B gene: P9 which consists of the sequence 5' CGGAATTCCTAGAAAAAATGGCAA  
20 ATG 3' (SEQ ID NO:15); P10 which consists of the sequence 5' GCTCTAGAATGA CCATAAGCTAGCCA 3' (SEQ ID NO:16); P11 which consists of the sequence 5' CGGAATTCGAGTTGGTAGAAAGGTGGA 3' (SEQ ID NO:17); P13 which consists of the sequence 5' CGGAATTCGGTTATTATCTTAAGGATG 3' (SEQ ID NO:18); and P14 which consists of the sequence 5' CGGAATTCTTGATAACTGGAT  
25 TTGTGAC 3' (SEQ ID NO:19). The amino acid sequence consisting of amino acid residues 1852 through 2362 of toxin B is listed in SEQ ID NO:20. The amino acid sequence consisting of amino acid residues 1755 through 2362 of toxin B is listed in SEQ ID NO:21.

*Clostridium difficile* VPI strain 10463 was obtained from the American Type Culture Collection (ATCC 43255) and grown under anaerobic conditions in brain-heart infusion medium (Becton Dickinson). High molecular-weight *C. difficile* DNA was isolated essentially as described [Wren and Tabaqchali (1987) J. Clin. Microbiol., 25:2402], except 1) 100 µg/ml proteinase K in 0.5% SDS was used to disrupt the bacteria and 2) cetytrimethylammonium bromide (CTAB) precipitation [as described by Ausubel *et al.*, Eds., *Current Protocols in Molecular Biology*, Vol. 2 (1989) Current Protocols] was used to remove carbohydrates from the cleared lysate. Briefly, after disruption of the bacteria with proteinase K and SDS, the solution is adjusted to approximately 0.7 M NaCl by the addition of a 1/7 volume of 5M NaCl. A 1/10 volume of CTAB/NaCl (10% CTAB in 0.7 M NaCl) solution was added and the solution was mixed thoroughly and incubated 10 min at 65°C. An equal volume of chloroform/isoamyl alcohol (24:1) was added and the phases were thoroughly mixed. The organic and aqueous phases were separated by centrifugation in a microfuge for 5 min. The aqueous supernatant was removed and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The phases were separated by centrifugation in a microfuge for 5 min. The supernatant was transferred to a fresh tube and the DNA was precipitated with isopropanol. The DNA precipitate was pelleted by brief centrifugation in a microfuge. The DNA pellet was washed with 70% ethanol to remove residual CTAB. The DNA pellet was then dried and redissolved in TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA). The integrity and yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

Toxin B fragments were cloned by PCR utilizing a proofreading thermostable DNA polymerase [native *Pfu* polymerase (Stratagene)]. The high fidelity of this polymerase reduces the mutation problems associated with amplification by error prone polymerases (*e.g.*, *Taq* polymerase). PCR amplification was performed using the PCR primer pairs P5 (SEQ ID NO:11) with P6 (SEQ ID NO:12) and P7 (SEQ ID NO:13) with P8 (SEQ ID NO:14) in 50 µl reactions containing 10 mM Tris-HCl pH8.3, 50

mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 0.2 µM each primer, and 50 ng *C. difficile* genomic DNA. Reactions were overlaid with 100 µl mineral oil, heated to 94°C for 4 min, 0.5µl native *Pfu* polymerase (Stratagene) was added, and the reactions were cycled 30 times at 94°C for 1 min, 50°C for 1 min, 72°C (2 min for each kb of sequence to be amplified), followed by 10 min at 72°C. Duplicate reactions were pooled, chloroform extracted, and ethanol precipitated. After washing in 70% ethanol, the pellets were resuspended in 50 µl TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA).

The P5/P6 amplification product was cloned into pUC19 as outlined below. 10µl aliquots of DNA were gel purified using the Prep-a-Gene kit (BioRad), and ligated to *Sma*I restricted pUC19 vector. Recombinant clones were isolated and confirmed by restriction digestion using standard recombinant molecular biology techniques (Sambrook *et al.*, 1989). Inserts from two independent isolates were identified in which the toxin B insert was oriented such that the vector *Bam*HI and *Sac*I sites were 5' and 3' oriented, respectively (pUCB10-1530). The insert-containing *Bam*HI/*Sac*I fragment was cloned into similarly cut pET23a-c vector DNA, and protein expression was induced in small scale cultures (5 ml) of identified clones.

Total protein extracts were isolated, resolved on SDS-PAGE gels, and toxin B protein identified by Western analysis utilizing a goat anti-toxin B affinity purified antibody (Tech Lab). Procedures for protein induction, SDS-PAGE, and Western blot analysis were performed as described in Williams *et al.* (1995), *supra*. In brief, 5 ml cultures of bacteria grown in 2XYT containing 100 µg/ml ampicillin containing the appropriate recombinant clone were induced to express recombinant protein by addition of IPTG to 1mM. The *E. coli* hosts used were: BL21(DE3) or BL21(DE3)LysS (Novagen) for pET plasmids.

Cultures were induced by the addition of IPTG to a final concentration of 1.0 mM when the cell density reached 0.5 OD<sub>600</sub>, and induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in microfuge), and resuspension of the

5 pelleted bacteria in 150 µl of 2X SDS-PAGE sample buffer (0.125 mM Tris-HCl pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 0.025% bromophenol blue; β-mercaptoethanol is added to 5% before use). The samples were heated to 95°C for 5 min, then cooled and 5 or 10 µls loaded on 7.5% SDS-PAGE gels. High molecular weight protein markers (BioRad) were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining the gels with Coomassie Blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. The MW of induced toxin B reactive protein allowed the integrity of the toxin B reading frame to be determined.

10 The pET23b recombinant (pPB10-1530) expressed high MW recombinant toxin B reactive protein, consistent with predicted values. This confirmed that frame terminating errors had not occurred during PCR amplification. A pET23b expression clone containing the 10-1750aa interval of the toxin B gene was constructed, by fusion of the *EcoRV*-*SpeI* fragment of the P7/P8 amplification product to the P5-*EcoRV* interval of the P5/P6 amplification product (see Figure 18) in pPB10-1530. The integrity of this clone (pPB10-1750) was confirmed by restriction mapping, and Western blot detection of expressed recombinant toxin B protein. Levels of induced protein from both pPB10-1530 and pPB10-1750 were too low to facilitate purification of usable amounts of recombinant toxin B protein. The remaining 1750-2360 aa interval was directly cloned into pMAL or pET expression vectors from the P7/P8 amplification product as described below.

## **b) Expression Of The Toxin B Gene**

### **i) Overview Of Expression Methodologies**

25 Fragments of the toxin B gene were expressed as either native or fusion proteins in *E. coli*. Native proteins were expressed in either the pET23a-c or pET16b expression vectors (Novagen). The pET23 vectors contain an extensive polylinker sequence in all three reading frames (a-c vectors) followed by a C-terminal poly-histidine repeat. The pET16b vector contains a N-terminal poly-histidine sequence

immediately 5' to a small polylinker. The poly-histidine sequence binds to Ni-Chelate columns and allows affinity purification of tagged target proteins [Williams *et al.* (1995), *supra*]. These affinity tags are small (10 aa for pET16b, 6 aa for pET23) allowing the expression and affinity purification of native proteins with only limited amounts of foreign sequences.

An N-terminal histidine-tagged derivative of pET16b containing an extensive cloning cassette was constructed to facilitate cloning of N-terminal poly-histidine tagged toxin B expressing constructs. This was accomplished by replacement of the promoter region of the pET23a and b vectors with that of the pET16b expression vector. Each vector was restricted with *Bgl*III and *Nde*I, and the reactions resolved on a 1.2 % agarose gel. The pET16b promoter region (contained in a 200 bp *Bgl*III-*Nde*I fragment) and the promoter-less pET23 a or b vectors were cut from the gel, mixed and Prep-A-Gene (BioRad) purified. The eluted DNA was ligated, and transformants screened for promoter replacement by *Nco*I digestion of purified plasmid DNA (the pET16b promoter contains this site, the pET23 promoter does not). These clones (denoted pETHisa or b) contain the pET16b promoter (consisting of a pT7-lac promoter, ribosome binding site and poly-histidine (10aa) sequence) fused at the *Nde*I site to the extensive pET23 polylinker.

All MBP fusion proteins were constructed and expressed in the pMAL<sup>TM</sup>-c or pMAL<sup>TM</sup>-p2 vectors (New England Biolabs) in which the protein of interest is expressed as a C-terminal fusion with MBP. All pET plasmids were expressed in either the BL21(DE3) or BL21(DE3)LysS expression hosts, while pMal plasmids were expressed in the BL21 host.

Large scale (500 mls to 1 liter) cultures of each recombinant were grown in 2X YT broth, induced, and soluble protein fractions were isolated as described [Williams, *et al.* (1995), *supra*]. The soluble protein extracts were affinity chromatographed to isolate recombinant fusion protein, as described [Williams *et al.*, (1995) *supra*]. In brief, extracts containing tagged pET fusions were chromatographed on a nickel chelate column, and eluted using imidazole salts or low pH (pH 4.0) as described by



the distributor (Novagen or Qiagen). Extracts containing soluble pMAL fusion protein were prepared and chromatographed in PBS buffer over an amylose resin (New England Biolabs) column, and eluted with PBS containing 10 mM maltose as described [Williams *et al.* (1995), *supra*].

5

## ii) Overview Of Toxin B Expression

In both large expression constructs described in (a) above, only low level (*i.e.*, less than 1 mg/liter of intact or nondegraded recombinant protein) expression of recombinant protein was detected. A number of expression constructs containing smaller fragments of the toxin B gene were then constructed, to determine if small  
10 regions of the gene can be expressed to high levels (*i.e.*, greater than 1 mg/liter intact protein) without extensive protein degradation. All were constructed by in frame fusions of convenient toxin B restriction fragments to either the pMAL-c, pET23a-c, pET16b or pETHisa-b expression vectors, or by engineering restriction sites at specific locations using PCR amplification [using the same conditions described in (a) above].  
15 In all cases, clones were verified by restriction mapping, and, where indicated, DNA sequencing.

Protein preparations from induced cultures of each of these constructs were analyzed, by SDS-PAGE, to estimate protein stability (Coomassie Blue staining) and immunoreactivity against anti-toxin B specific antiserum (Western analysis). Higher  
20 levels of intact (*i.e.*, nondegraded), full length fusion proteins were observed with the smaller constructs as compared with the larger recombinants, and a series of expression constructs spanning the entire toxin B gene were constructed (Figures 18, 19 and 20 and Table 23).

Constructs that expressed significant levels of recombinant toxin B protein  
25 (greater than 1 mg/liter intact recombinant protein) in *E. coli* were identified and a series of these clones that spans the toxin B gene are shown in Figure 19 and summarized in Table 23. These clones were utilized to isolate pure toxin B recombinant protein from the entire toxin B gene. Significant protein yields were

obtained from pMAL expression constructs spanning the entire toxin B gene, and yields of full length soluble fusion protein ranged from an estimated 1 mg/liter culture (pMB1100-1530) to greater than 20 mg/liter culture (pMB1750-2360).

5 Representative purifications of MBP and poly-histidine-tagged toxin B recombinants are shown in Figures 21 and 22. Figure 21 shows a Coomassie Blue stained 7.5% SDS-PAGE gel on which various protein samples extracted from bacteria harboring pMB1850-2360 were electrophoresed. Samples were loaded as follows: Lane 1: protein extracted from uninduced culture; Lane 2: induced culture protein; Lane 3: total protein from induced culture after sonication; Lane 4: soluble protein; 10 and Lane 5: eluted affinity purified protein. Figure 22 depicts the purification of recombinant proteins expressed in bacteria harboring either pPB1850-2360 (Lanes 1-3) or pPB1750-2360 (Lanes 4-6). Samples were loaded as follows: uninduced total protein (Lanes 1 and 4); induced total protein (Lanes 2 and 5); and eluted affinity purified protein (Lanes 3 and 6). The broad range molecular weight protein markers (BioRad) are shown in Lane 7. 15

Thus, although high level expression was not attained using large expression constructs from the toxin B gene, usable levels of recombinant protein were obtained by isolating induced protein from a series of smaller pMAL expression constructs that span the entire toxin B gene.

20 These results represent the first demonstration of the feasibility of expressing recombinant toxin B protein to high levels in *E. coli*. As well, expression of small regions of the putative ligand binding domain (repeat region) of toxin B as native protein yielded insoluble protein, while large constructs, or fusions to MBP were soluble (Figure 19), demonstrating that specific methodologies are necessary to 25 produce soluble fusion protein from this interval.

### iii) Clone Construction And Expression

#### Details

5 A portion of the toxin B gene containing the toxin B repeat region [amino acid residues 1852-2362 of toxin B (SEQ ID NO:20)] was isolated by PCR amplification of this interval of the toxin B gene from *C. difficile* genomic DNA. The sequence, and location within the toxin B gene, of the two PCR primers [P7 (SEQ ID NO:13) and P8 (SEQ ID NO:14)] used to amplify this region are shown in Figure 18.

10 DNA from the PCR amplification was purified by chloroform extraction and ethanol precipitation as described above. The DNA was restricted with *SpeI*, and the cleaved DNA was resolved by agarose gel electrophoresis. The restriction digestion with *SpeI* cleaved the 3.6 kb amplification product into a 1.8 kb doublet band. This doublet band was cut from the gel and mixed with appropriately cut, gel purified pMALc or pET23b vector. These vectors were prepared by digestion with *HindIII*, filling in the overhanging ends using the Klenow enzyme, and cleaving with *XbaI* (pMALc) or *NheI* (pET23b). The gel purified DNA fragments were purified using the Prep-A-Gene kit (BioRad) and the DNA was ligated, transformed and putative recombinant clones analyzed by restriction mapping.

20 pET and pMal clones containing the toxin B repeat insert (aa interval 1750-2360 of toxin B) were verified by restriction mapping, using enzymes that cleaved specific sites within the toxin B region. In both cases fusion of the toxin B *SpeI* site with either the compatible *XbaI* site (pMal) or compatible *NheI* site (pET) is predicted to create an in frame fusion. This was confirmed in the case of the pMB1750-2360 clone by DNA sequencing of the clone junction and 5' end of the toxin B insert using a MBP specific primer (New England Biolabs). In the case of the pET construct, the fusion of the blunt ended toxin B 3' end to the filled *HindIII* site should create an in-frame fusion with the C-terminal poly-histidine sequence in this vector. The pPB1750-2360 clone selected had lost, as predicted, the *HindIII* site at this clone junction; this eliminated the possibility that an additional adenosine residue was added to the 3' end of the PCR product by a terminal transferase activity of the *Pfu* polymerase, since

fusion of this adenosine residue to the filled *Hind*III site would regenerate the restriction site (and was observed in several clones).

One liter cultures of each expression construct were grown, and fusion protein purified by affinity chromatography on either an amylose resin column (pMAL constructs; resin supplied by New England Biolabs) or Ni-chelate column (pET constructs; resin supplied by Qiagen or Novagen) as described [Williams *et al.* (1995), *supra*]. The integrity and purity of the fusion proteins were determined by Coomassie staining of SDS-PAGE protein gels as well as Western blot analysis with either an affinity purified goat polyclonal antiserum (Tech Lab), or a chicken polyclonal PEG prep, raised against the toxin B protein (CTB) as described above in Example 8. In both cases, affinity purification resulted in yields in excess of 20 mg protein per liter culture, of which greater than 90% was estimated to be full-length recombinant protein. It should be noted that the poly-histidine affinity tagged protein was released from the Qiagen Ni-NTA resin at low imidazole concentration (60 mM), necessitating the use of a 40 mM imidazole rather than a 60 mM imidazole wash step during purification.

A periplasmically secreted version of pMB1750-2360 was constructed by replacement of the promoter and MBP coding region of this construct with that from a related vector (pMAL<sup>TM</sup>-p2; New England Biolabs) in which a signal sequence is present at the N-terminus of the MBP, such that fusion protein is exported. This was accomplished by substituting a *Bgl*II-*Eco*RV promoter fragment from pMAL-p2 into pMB1750-2360. The yields of secreted, affinity purified protein (recovered from osmotic shock extracts as described by Riggs in *Current Protocols in Molecular Biology*, Vol. 2, Ausubel, *et al.*, Eds. (1989), Current Protocols, pp. 16.6.1 - 16.6.14] from this vector (pMBp1750-2360) were 6.5 mg/liter culture, of which 50% was estimated to be full-length fusion protein.

The interval was also expressed in two non-overlapping fragments. pMB1750-1970 was constructed by introduction of a frameshift into pMB1750-2360, by restriction with *Hind*III, filling in the overhanging ends and religation of the plasmid.

Recombinant clones were selected by loss of the *Hind*III site, and further restriction map analysis. Recombinant protein expression from this vector was more than 20 mg/liter of greater than 90% pure protein.

5       The complementary region was expressed in pMB1970-2360. This construct was created by removal of the 1750-1970 interval of pMB1750-2360. This was accomplished by restriction of this plasmid with *Eco*RI (in the pMalc polylinker 5' to the insert) and III, filling in the overhanging ends, and religation of the plasmid. The resultant plasmid, pMB1970-2360, was made using both intracellularly and secreted versions of the pMB1750-2360 vector.

10       No fusion protein was secreted in the pMBp1970-2360 version, perhaps due to a conformational constraint that prevents export of the fusion protein. However, the intracellularly expressed vector produced greater than 40mg/liter of greater than 90% full-length fusion protein.

15       Constructs to precisely express the toxin B repeats in either pMalc (pMB1850-2360) or pET16b (pPB1850-2360) were constructed as follows. The DNA interval including the toxin B repeats was PCR amplified as described above utilizing PCR primers P14 (SEQ ID NO:19) and P8 (SEQ ID NO:14). Primer P14 adds a *Eco*RI site immediately flanking the start of the toxin B repeats.

20       The amplified fragment was cloned into the pT7 Blue T-vector (Novagen) and recombinant clones in which single copies of the PCR fragment were inserted in either orientation were selected (pT71850-2360) and confirmed by restriction mapping. The insert was excised from two appropriately oriented independently isolated pT71850-2360 plasmids, with *Eco*RI (5' end of repeats) and *Pst*I (in the flanking polylinker of the vector), and cloned into *Eco*RI/*Pst*I cleaved pMalc vector. The resulting construct  
25       (pMB1850-2360) was confirmed by restriction analysis, and yielded 20 mg/l of soluble fusion protein [greater than 90% intact (*i.e.*, nondegraded)] after affinity chromatography. Restriction of this plasmid with *Hind*III and religation of the vector resulted in the removal of the 1970-2360 interval. The resultant construct (pMB1850-1970) expressed greater than 70 mg/liter of 90% full length fusion protein.

The pPB1850-2360 construct was made by cloning a *EcoRI* (filled with Klenow)-*BamHI* fragment from a pT71850-2360 vector (opposite orientation to that used in the pMB1850-2360 construction) into *NdeI* (filled)/*BamHI* cleaved pET16b vector. Yields of affinity purified soluble fusion protein were 15 mg/liter, of greater than 90% full length fusion protein.

Several smaller expression constructs from the 1750-2070 interval were also constructed in His-tagged pET vectors, but expression of these plasmids in the BL21 (DE3) host resulted in the production of high levels of mostly insoluble protein (see Table 23 and Figure 19). These constructs were made as follows.

pPB1850-1970 was constructed by cloning a *BglIII-HindIII* fragment of pPB1850-2360 into *BglIII/HindIII* cleaved pET23b vector. pPB1850-2070 was constructed by cloning a *BglIII-PvuII* fragment of pPB1850-2360 into *BglIII/HincII* cleaved pET23b vector. pPB1750-1970(c) was constructed by removal of the internal *HindIII* fragment of a pPB1750-2360 vector in which the vector *HindIII* site was regenerated during cloning (presumably by the addition of an A residue to the amplified PCR product by terminal transferase activity of *Pfu* polymerase). The pPB1750-1970(n) construct was made by insertion of the insert containing the *NdeI-HindIII* fragment of pPB1750-2360 into identically cleaved pETHisb vector. All constructs were confirmed by restriction digestion.

An expression construct that directs expression of the 10-470 aa interval of toxin B was constructed in the pMalc vector as follows. A *NheI* (a site 5' to the insert in the pET23 vector)-*AflIII* (filled) fragment of the toxin B gene from pPB10-1530 was cloned into *XbaI* (compatible with *NheI*)/*HindIII* (filled) pMalc vector. The integrity of the construct (pMB10-470) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer (New England Biolabs). However, all expressed protein was degraded to the MBP monomer MW.

A second construct spanning this interval (aa 10-470) was constructed by cloning the PCR amplification product from a reaction containing the P9 (SEQ ID NO:15) and P10 (SEQ ID NO:16) primers (Figure 18) into the pETHisa vector. This

was accomplished by cloning the PCR product as an *EcoRI*-blunt fragment into *EcoRI*-*HincII* restricted vector DNA; recombinant clones were verified by restriction mapping. Although this construct (pPB10-520) allowed expression and purification (utilizing the N-terminal polyhistidine affinity tag) of intact fusion protein, yields were  
5 estimated at less than 500 µg per liter culture.

Higher yield of recombinant protein from this interval (aa 10-520) were obtained by expression of the interval in two overlapping clones. The 10-330aa interval was cloned in both pETHisa and pMalc vectors, using the *BamHI*-*AflIII* (filled) DNA fragment from pPB10-520. This fragment was cloned into *BamHI*-  
10 *HindIII* (filled) restricted pMalc or *BamHI*-*HincII* restricted pETHisa vector. Recombinant clones were verified by restriction mapping. High level expression of either insoluble (pET) or soluble (pMal) fusion protein was obtained. Total yields of fusion protein from the pMB10-330 construct (Figure 18) were 20 mg/liter culture, of which 10% was estimated to be full-length fusion protein. Although yields of this  
15 interval were higher and >90% full-length recombinant protein produced when expressed from the pET construct, the pMal fusion was utilized since the expressed protein was soluble and thus more likely to have the native conformation.

The pMB260-520 clone was constructed by cloning *EcoRI*-*XbaI* cleaved amplified DNA from a PCR reaction containing the P11 (SEQ ID NO:17) and P10  
20 (SEQ ID NO:16) DNA primers (Figure 18) into similarly restricted pMalc vector. Yields of affinity purified protein were 10 mg/liter, of which approximately 50% was estimated to be full-length recombinant protein.

The aa510-1110 interval was expressed as described below. This entire interval was expressed as a pMal fusion by cloning the *NheI*-*HindIII* fragment of pUCB10-  
25 1530 into *XbaI*-*HindIII* cleaved pMalc vector. The integrity of the construct (pMB510-1110) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer. The yield of affinity purified protein was 25 mg/liter culture, of which 5% was estimated to be full-length fusion protein (1 mg/liter).

To attempt to obtain higher yields, this region was expressed in two fragments (aa510-820, and 820-1110) in the pMalc vector. The pMB510-820 clone was constructed by insertion of a *SacI* (in the pMalc polylinker 5' to the insert)-*HpaI* DNA fragment from pMB510-1110 into *SacI/StuI* restricted pMalc vector. The pMB820-1110 vector was constructed by insertion of the *HpaI-HindIII* fragment of pUCB10-1530 into *BamHI* (filled)/*HindIII* cleaved pMalc vector. The integrity of these constructs were verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer.

Recombinant protein expressed from the pMB510-820 vector was highly unstable. However, high levels (20 mg/liter) of >90% full-length fusion protein were obtained from the pMB820-1105 construct. The combination of partially degraded pMB510-1110 protein (enriched for the 510-820 interval) with the pMB820-1110 protein provides usable amounts of recombinant antigen from this interval.

The aa1100-1750 interval was expressed as described below. The entire interval was expressed in the pMalc vector from a construct in which the *AccI*(filled)-*SpeI* fragment of pPB10-1750 was inserted into *StuI/XbaI* (*XbaI* is compatible with *SpeI*; *StuI* and filled *AccI* sites are both blunt ended) restricted pMalc. The integrity of this construct (pMB1100-1750) was verified by restriction mapping and DNA sequencing of the clone junction using a MBP specific DNA primer. Although 15 mg/liter of affinity purified protein was isolated from cells harboring this construct, the protein was greater than 99% degraded to MBP monomer size.

A smaller derivative of pMB1100-1750 was constructed by restriction of pMB1100-1750 with *AflII* and *SalI* (in the pMalc polylinker 3' to the insert), filling in the overhanging ends, and religating the plasmid. The resultant clone (verified by restriction digestion and DNA sequencing) has deleted the aa1530-1750 interval, was designated pMB1100-1530. pMB1100-1530 expressed recombinant protein at a yield of greater than 40 mg/liter, of which 5% was estimated to be full-length fusion protein.



Three constructs were made to express the remaining interval. Initially, a *Bsp*HI (filled)-*Spe*I fragment from pPB10-1750 was cloned into *Eco*RI(filled)/*Xba*I cleaved pMalc vector. The integrity of this construct (pMB1570-1750) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer. Expression of recombinant protein from this plasmid was very low, approximately 3 mg affinity purified protein per liter, and most was degraded to MBP monomer size. This region was subsequently expressed from a PCR amplified DNA fragment. A PCR reaction utilizing primers P13 [SEQ ID NO:18; P13 was engineered to introduce an *Eco*RI site 5' to amplified toxin B sequences] and P8 (SEQ ID NO:14) was performed on *C. difficile* genomic DNA as described above. The amplified fragment was cleaved with *Eco*RI and *Spe*I, and cloned into *Eco*RI/*Xba*I cleaved pMalc vector. The resultant clone (pMB1530-1750) was verified by restriction map analysis, and recombinant protein was expressed and purified. The yield was greater than 20 mg protein per liter culture and it was estimated that 25% was full-length fusion protein; this was a significantly higher yield than the original pMB1570-1750 clone. The insert of pMB1530-1750 (in a *Eco*RI-*Sal*I fragment) was transferred to the pETHisa vector (*Eco*RI/*Xho*I cleaved, *Xho*I and *Sal*I ends are compatible). No detectable fusion protein was purified on Ni-Chelate columns from soluble lysates of cells induced to express fusion protein from this construct.

TABLE 23

Summary Of Toxin B Expression Constructs<sup>a</sup>

Clone	Affinity Tag	Yield (mg/liter)	% Full Length
pPB10-1750	none	low (estimated from Western blot hyb.)	?
pPB10-1530	none	low (as above)	?
pMB10-470	MBP	15mg/l	0%
pPB10-520	poly-his	0.5mg/l	20%
pPB10-330	poly-his	>20mg/l (insoluble)	90%
pMB10-330	MBP	20mg/l	10%
pMB260-520	MBP	10mg/l	50%

TABLE 23

Summary Of Toxin B Expression Constructs<sup>a</sup>

Clone	Affinity Tag	Yield (mg/liter)	% Full Length
<i>pMB510-1110</i>	<i>MBP</i>	25mg/l	5%
pMB510-820	MBP	degraded (by Western blot hyb)	
<i>pMB820-1110</i>	<i>MBP</i>	20mg/l	90%
pMB1100-1750	MBP	15mg/l	0%
<i>pMB1100-1530</i>	<i>MBP</i>	40mg/l	5%
pMB1570-1750	MBP	3mg/l	<5%
pPB1530-1750	poly-his	no purified protein detected	?
<i>pMB1530-1750</i>	<i>MBP</i>	20mg/l	25%
<i>pMB1750-2360</i>	<i>MBP</i>	>20mg/l	>90%
pMBp1750-2360	MBP	6.5mg/l (secreted)	50%
pPB1750-2360	poly-his	>20mg/l	>90%
pMB1750-1970	MBP	>20mg/l	>90%
pMB1970-2360	MBP	40mg/l	>90%
pMBp1970-2360	MBP	(no secretion)	NA
pMB1850-2360	MBP	20mg/l	>90%
pPB1850-2360	poly-his	15mg/l	>90%
pMB1850-1970	MBP	70mg/l	>90%
pPB1850-1970	poly-his	>10mg/l (insoluble)	>90%
pPB1850-2070	poly-his	>10mg/l (insoluble)	>90%
pPB1750-1970(c)	poly-his	>10mg/l (insoluble)	>90%
pPB1750-1970(n)	poly-his	>10mg/l (insoluble)	>90%

<sup>a</sup> Clones in italics are clones currently utilized to purify recombinant protein from each selected interval.

## EXAMPLE 19

### Identification, Purification And Induction Of Neutralizing Antibodies Against Recombinant *C. difficile* Toxin B Protein

To determine whether recombinant toxin B polypeptide fragments can generate neutralizing antibodies, typically animals would first be immunized with recombinant proteins and anti-recombinant antibodies are generated. These anti-recombinant protein antibodies are then tested for neutralizing ability *in vivo* or *in vitro*. Depending on the immunogenic nature of the recombinant polypeptide, the generation of high-titer antibodies against that protein may take several months. To accelerate this process and identify which recombinant polypeptide(s) may be the best candidate to generate neutralizing antibodies, depletion studies were performed. Specifically, recombinant toxin B polypeptide were pre-screened by testing whether they have the ability to bind to protective antibodies from a CTB antibody preparation and hence deplete those antibodies of their neutralizing capacity. Those recombinant polypeptides found to bind CTB, were then utilized to generate neutralizing antibodies. This Example involved: a) identification of recombinant sub-regions within toxin B to which neutralizing antibodies bind; b) identification of toxin B sub-region specific antibodies that neutralize toxin B *in vivo*; and c) generation and evaluation of antibodies reactive to recombinant toxin B polypeptides.

#### a) Identification Of Recombinant Sub-Regions Within Toxin B To Which Neutralizing Antibodies Bind

Sub-regions within toxin B to which neutralizing antibodies bind were identified by utilizing recombinant toxin B proteins to deplete protective antibodies from a polyclonal pool of antibodies against native *C. difficile* toxin B. An *in vivo* assay was developed to evaluate protein preparations for the ability to bind neutralizing

antibodies. Recombinant proteins were first pre-mixed with antibodies directed against native toxin B (CTB antibody; see Example 8) and allowed to react for one hour at 37°C. Subsequently, *C. difficile* toxin B (CTB; Tech Lab) was added at a concentration lethal to hamsters and incubated for another hour at 37°C. After incubation this mixture was injected intraperitoneally (IP) into hamsters. If the recombinant polypeptide contains neutralizing epitopes, the CTB antibodies will lose its ability to protect the hamsters against death from CTB. If partial or complete protection occurs with the CTB antibody-recombinant mixture, that recombinant contains only weak or non-neutralizing epitopes of toxin B. This assay was performed as follows.

Antibodies against CTB were generated in egg laying Leghorn hens as described in Example 8. The lethal dosage ( $LD_{100}$ ) of *C. difficile* toxin B when delivered I.P. into 40g female Golden Syrian hamsters (Charles River) was determined to be 2.5 to 5 µg. Antibodies generated against CTB and purified by PEG precipitation could completely protect the hamsters at the I.P. dosage determined above. The minimal amount of CTB antibody needed to afford good protection against 5 µg of CTB when injected I.P. into hamsters was also determined (1X PEG prep). These experiments defined the parameters needed to test whether a given recombinant protein could deplete protective CTB antibodies.

The cloned regions tested for neutralizing ability cover the entire toxin B gene and were designated as Intervals (INT) 1 through 5 (see Figure 19). Approximately equivalent final concentrations of each recombinant polypeptide were tested. The following recombinant polypeptides were used: 1) a mixture of intervals 1 and 2 (INT-1, 2); 2) a mixture of Intervals 4 and 5 (INT-4, 5) and 3) Interval 3 (INT-3). Recombinant proteins (each at about 1 mg total protein) were first preincubated with a final CTB antibody concentration of 1X [*i.e.*, pellet dissolved in original yolk volume as described in Example 1(c)] in a final volume of 5 mls for 1 hour at 37°C. Twenty-

five µg of CTB (at a concentration of 5 µg/ml), enough CTB to kill 5 hamsters, was then added and the mixture was then incubated for 1 hour at 37°C. Five, 40g female hamsters (Charles River) in each treatment group were then each given 1 ml of the mixture I.P. using a tuberculin syringe with a 27 gauge needle. The results of this experiment are shown in Table 24.

TABLE 24

Binding Of Neutralizing Antibodies By INT 3 Protein

Treatment Group <sup>1</sup>	Number Of Animals Alive	Number Of Animals Dead
CTB antibodies	3	2
CTB antibodies + INT1,2	3	2
CTB antibodies + INT4,5	3	2
CTB antibodies + INT 3	0	5

<sup>1</sup> *C. difficile* toxin B (CTB) was added to each group.

As shown in Table 24, the addition of recombinant proteins from INT-1, 2 or INT-4, 5 had no effect on the *in vivo* protective ability of the CTB antibody preparation compared to the CTB antibody preparation alone. In contrast, INT-3 recombinant polypeptide was able to remove all of the toxin B neutralizing ability of the CTB antibodies as demonstrated by the death of all the hamsters in that group.

The above experiment was repeated, using two smaller expressed fragments (pMB 1750-1970 and pMB 1970-2360, see Figure 19) comprising the INT-3 domain to determine if that domain could be further subdivided into smaller neutralizing epitopes. In addition, full-length INT-3 polypeptide expressed as a nickel tagged protein (pPB1750-2360) was tested for neutralizing ability and compared to the original INT-3 expressed MBP fusion (pMB1750-2360). The results are shown in Table 25.

TABLE 25

Removal Of Neutralizing Antibodies By Repeat Containing Proteins

Treatment Group <sup>1</sup>	Number Of Animals Alive	Number Of Animals Dead
CTB antibodies	5	0
CTB antibodies + pPB1750-2360	0	5
CTB antibodies + pMB1750-2360	0	5
CTB antibodies + pMB1970-2360	3	2
CTB antibodies + pMB1750-1970	2	3

<sup>1</sup> *C. difficile* toxin B (CTB) was added to each group.

The results summarized in Table 25 indicate that the smaller polypeptide fragments within the INT-3 domain, pMB1750-1970 and pMB1970-2360, partially lose the ability to bind to and remove neutralizing antibodies from the CTB antibody pool. These results demonstrate that the full length INT-3 polypeptide is required to completely deplete the CTB antibody pool of neutralizing antibodies. This experiment also shows that the neutralization epitope of INT-3 can be expressed in alternative vector systems and the results are independent of the vector utilized or the accompanying fusion partner.

Other Interval 3 specific proteins were subsequently tested for the ability to remove neutralizing antibodies within the CTB antibody pool as described above. The Interval 3 specific proteins used in these studies are summarized in Figure 23. In Figure 23 the following abbreviations are used: pP refers to the pET23 vector; pM refers to the pMALc vector; B refers to toxin B; the numbers refer to the amino acid interval expressed in the clone. The solid black ovals represent the MBP; and HHH represents the poly-histidine tag.

Only recombinant proteins comprising the entire toxin B repeat domain (pMB1750-2360, pPB1750-2360 and pPB1850-2360) can bind and completely remove neutralizing antibodies from the CTB antibody pool. Recombinant proteins comprising only a portion of the toxin B repeat domain were not capable of completely removing

neutralizing antibodies from the CTB antibody pool (pMB1750-1970 and pMB1970-2360 could partially remove neutralizing antibodies while pMB1850-1970 and pPB1850-2070 failed to remove any neutralizing antibodies from the CTB antibody pool).

5           The above results demonstrate that only the complete ligand binding domain (repeat region) of the toxin B gene can bind and completely remove neutralizing antibodies from the CTB antibody pool. These results demonstrate that antibodies directed against the entire toxin B repeat region are necessary for *in vivo* toxin neutralization (see Figure 23; only the recombinant proteins expressed by the  
10       pMB1750-2360, pPB1750-2360 and pPB1850-2360 vectors are capable of completely removing the neutralizing antibodies from the CTB antibody pool).

          These results represent the first indication that the entire repeat region of toxin B would be necessary for the generation of antibodies capable of neutralizing toxin B, and that sub-regions may not be sufficient to generate maximal titers of neutralizing  
15       antibodies.

**b)       Identification Of Toxin B Sub-Region Specific  
          Antibodies That Neutralize Toxin B *In Vivo***

          To determine if antibodies directed against the toxin B repeat region are  
20       sufficient for neutralization, region specific antibodies within the CTB antibody preparation were affinity purified, and tested for *in vivo* neutralization. Affinity columns containing recombinant toxin B repeat proteins were made as described below. A separate affinity column was prepared using each of the following recombinant toxin B repeat proteins: pPB1750-2360, pPB1850-2360, pMB1750-1970 and pMB1970-2360.

25           For each affinity column to be made, four ml of PBS-washed Actigel resin (Sterogene) was coupled overnight at room temperature with 5-10 mg of affinity purified recombinant protein (first extensively dialyzed into PBS) in 15 ml tubes (Falcon) containing a 1/10 final volume Ald-coupling solution (1 M sodium cyanoborohydride). Aliquots of the supernatants from the coupling reactions, before

and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, in all cases greater than 30% coupling efficiencies were estimated. The resins were poured into 10 ml columns (BioRad), washed extensively with PBS, pre-eluted with 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0) and reequilibrated in PBS. The columns were stored at 4°C.

Aliquots of a CTB IgY polyclonal antibody preparation (PEG prep) were affinity purified on each of the four columns as described below. The columns were hooked to a UV monitor (ISCO), washed with PBS and 40 ml aliquots of a 2X PEG prep (filter sterilized using a 0.45  $\mu$  filter) were applied. The columns were washed with PBS until the baseline value was re-established. The columns were then washed with BBStween to elute nonspecifically binding antibodies, and reequilibrated with PBS. Bound antibody was eluted from the column in 4M guanidine-HCl (in 10mM Tris-HCl, pH8.0). The eluted antibody was immediately dialyzed against a 100-fold excess of PBS at 4°C for 2 hrs. The samples were then dialyzed extensively against at least 2 changes of PBS, and affinity purified antibody was collected and stored at 4°C. The antibody preparations were quantified by UV absorbance. The elution volumes were in the range of 4-8 ml. All affinity purified stocks contained similar total antibody concentrations, ranging from 0.25-0.35% of the total protein applied to the columns.

The ability of the affinity purified antibody preparations to neutralize toxin B *in vivo* was determined using the assay outlined in a) above. Affinity purified antibody was diluted 1:1 in PBS before testing. The results are shown in Table 26.

In all cases similar levels of toxin neutralization was observed, such that lethality was delayed in all groups relative to preimmune controls. This result demonstrates that antibodies reactive to the repeat region of the toxin B gene are sufficient to neutralize toxin B *in vivo*. The hamsters will eventually die in all groups, but this death is maximally delayed with the CTB PEG prep antibodies. Thus neutralization with the affinity purified (AP) antibodies is not as complete as that observed with the CTB prep before affinity chromatography. This result may be due to loss of activity during guanidine denaturation (during the elution of the antibodies



from the affinity column) or the presence of antibodies specific to other regions of the toxin B gene that can contribute to toxin neutralization (present in the CTB PEG prep).

TABLE 26

Neutralization Of Toxin B By Affinity Purified Antibodies

Treatment group <sup>a</sup>	Number Animals Alive <sup>b</sup>	Number Animals Dead <sup>b</sup>
Preimmune <sup>1</sup>	0	5
CTB <sup>1</sup> ; 400 µg	5	0
CTB (AP on pPB1750-2360); <sup>2</sup> 875 µg	5	0
CTB (AP on pMB1750-1970); <sup>2</sup> 875 µg	5	0
CTB (AP on pMB1970-2360); <sup>2</sup> 500 µg	5	0

<sup>a</sup> *C. difficile* toxin B (CTB) (Tech Lab; at 5 µg/ml, 25 µg total) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as either: <sup>1</sup>4X antibody PEG prep or <sup>2</sup>affinity purified (AP) antibody (from CTB PEG prep, on indicated columns). The amount of specific antibody in each prep is indicated; the amount is directly determined for affinity purified preps and is estimated for the 4X CTB as described in Example 15.

<sup>b</sup> The numbers in each group represent numbers of hamsters dead or alive, 2 hr post IP administration of toxin/antibody mixture.

The observation that antibodies affinity purified against the non-overlapping pMB1750-1970 and pMB1970-2360 proteins neutralized toxin B raised the possibility that either 1) antibodies specific to repeat sub-regions are sufficient to neutralize toxin B or 2) sub-region specific proteins can bind most or all repeat specific antibodies present in the CTB polyclonal pool. This would likely be due to conformational similarities between repeats, since homology in the primary amino acid sequences between different repeats is in the range of only 25-75% [Eichel-Streiber, *et al.* (1992) Molec. Gen. Genetics 233:260]. These possibilities were tested by affinity chromatography.

The CTB PEG prep was sequentially depleted 2X on the pMB1750-1970 column; only a small elution peak was observed after the second chromatography,

indicating that most reactive antibodies were removed. This interval depleted CTB preparation was then chromatographed on the pPB1850-2360 column; no antibody bound to the column. The reactivity of the CTB and CTB (pMB1750-1970 depleted) preps to pPB1750-2360, pPB1850-2360, pMB1750-1970 and pMB1970-2360 proteins was then determined by ELISA using the protocol described in Example 13(c).  
5 Briefly, 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were coated with recombinant protein by adding 100  $\mu$ l volumes of protein at 1-2  $\mu$ g/ml in PBS containing 0.005% thimerosal to each well and incubating overnight at 4°C. The next morning, the coating suspensions were decanted and the wells were washed three times  
10 using PBS. In order to block non-specific binding sites, 100  $\mu$ l of 1.0% BSA (Sigma) in PBS (blocking solution) was then added to each well, and the plates were incubated for 1 hr. at 37°C. The blocking solution was decanted and duplicate samples of 150  $\mu$ l of diluted antibody was added to the first well of a dilution series. The initial testing serum dilution was (1/200 for CTB prep, (the concentration of depleted CTB was standardized by OD<sub>280</sub>) in blocking solution containing 0.5% Tween 20, followed  
15 by 5-fold serial dilutions into this solution. This was accomplished by serially transferring 30  $\mu$ l aliquots to 120  $\mu$ l buffer, mixing, and repeating the dilution into a fresh well. After the final dilution, 30  $\mu$ l was removed from the well such that all wells contained 120  $\mu$ l final volume. A total of 5 such dilutions were performed (4  
20 wells total). The plates were incubated for 1 hr at 37°C. Following this incubation, the serially diluted samples were decanted and the wells were washed three times using PBS containing 0.5% Tween 20 (PBST), followed by two 5 min washes using BBS-Tween and a final three washes using PBST. To each well, 100  $\mu$ l of 1/1000 diluted secondary antibody [rabbit anti-chicken IgG alkaline phosphatase (Sigma) diluted in  
25 blocking solution containing 0.5% Tween 20] was added, and the plate was incubated 1 hr at 37°C. The conjugate solutions were decanted and the plates were washed 6 times in PBST, then once in 50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH 9.5. The plates were developed by the addition of 100  $\mu$ l of a solution containing 1 mg/ml para-nitro phenyl phosphate (Sigma) dissolved in 50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH9.5 to each  
30 well. The plates were then incubated at room temperature in the dark for 5-45 min.

The absorbency of each well was measured at 410 nm using a Dynatech MR 700 plate reader.

As predicted by the affinity chromatography results, depletion of the CTB prep on the pMB1750-1970 column removed all detectable reactivity to the pMB1970-2360 protein. The reciprocal purification of a CTB prep that was depleted on the pMB1970-2360 column yielded no bound antibody when chromatographed on the pMB1750-1970 column. These results demonstrate that all repeat reactive antibodies in the CTB polyclonal pool recognize a conserved structure that is present in non-overlapping repeats. Although it is possible that this conserved structure represents rare conserved linear epitopes, it appears more likely that the neutralizing antibodies recognize a specific protein conformation. This conclusion was also suggested by the results of Western blot hybridization analysis of CTB reactivity to these recombinant proteins.

Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of each recombinant protein, were probed with the CTB polyclonal antibody preparation. The blots were prepared and developed with alkaline phosphatase as described in Example 3. The results are shown in Figure 24.

Figure 24 depicts a comparison of immunoreactivity of IgY antibody raised against either native or recombinant toxin B antigen. Equal amounts of pMB1750-1970 (lane 1), pMB1970-2360 (lane 2), pPB1850-2360 (lane 3) as well as a serial dilution of pPB1750-2360 (lanes 4-6 comprising 1X, 1/10X and 1/100X amounts, respectively) proteins were loaded in duplicate and resolved on a 7.5% SDS-PAGE gel. The gel was blotted and each half was hybridized with PEG prep IgY antibodies from chickens immunized with either native CTB or pPB1750-2360 protein. Note that the full-length pMB1750-1970 protein was identified only by antibodies reactive to the recombinant protein (arrows).

Although the CTB prep reacts with the pPB1750-2360, pPB1850-2360, and pMB1970-2360 proteins, no reactivity to the pMB1750-1970 protein was observed (Figure 24). Given that all repeat reactive antibodies can be bound by this protein during affinity chromatography, this result indicates that the protein cannot fold

properly on Western blots. Since this eliminates all antibody reactivity, it is unlikely that the repeat reactive antibodies in the CTB prep recognize linear epitopes. This may indicate that in order to induce protective antibodies, recombinant toxin B protein will need to be properly folded.

5                    **c)        Generation And Evaluation Of Antibodies Reactive To  
                         Recombinant Toxin B Polypeptides**

**i)        Generation Of Antibodies Reactive To  
                         Recombinant Toxin B Proteins**

10        Antibodies against recombinant proteins were generated in egg laying Leghorn hens as described in Example 13. Antibodies were raised [using Freund's adjuvant (Gibco) unless otherwise indicated] against the following recombinant proteins: 1) a mixture of Interval 1+2 proteins (see Figure 18); 2) a mixture of interval 4 and 5 proteins (see Figure 18); 3) pMB1970-2360 protein; 4) pPB1750-2360 protein; 5) pMB1750-2360; 6) pMB1750-2360 [Titermax adjuvant (Vaxcell)]; 7) pMB1750-2360 [Gerbu adjuvant (Biotech)]; 8) pMBp1750-2360 protein; 9) pPB1850-2360; and 10) pMB1850-2360.

15                Chickens were boosted at least 3 times with recombinant protein until ELISA reactivity [using the protocol described in b) above with the exception that the plates were coated with pPB1750-2360 protein] of polyclonal PEG preps was at least equal to that of the CTB polyclonal antibody PEG prep. ELISA titers were determined for  
20        the PEG preps from all of the above immunogens and were found to be comparable ranging from 1:12500 to 1:62500. High titers were achieved in all cases except in 6) pMB1750-2360 in which strong titers were not observed using the Titermax adjuvant, and this preparation was not tested further.

**ii) Evaluation Of Antibodies Reactive To  
Recombinant Proteins By Western Blot  
Hybridization**

Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of recombinant protein (pMB1750-1970, pPB1850-2360, and pMB1970-2360 proteins and a serial dilution of the pPB1750-2360 to allow quantification of reactivity), were probed with the CTB, pPB1750-2360, pMB1750-2360 and pMB1970-2360 polyclonal antibody preparations (from chickens immunized using Freund's adjuvant). The blots were prepared and developed with alkaline phosphatase as described above in b).

As shown in Figure 24, the CTB and pMB1970-2360 preps reacted strongly with the pPB1750-2360, pPB1850-2360, and pMB1970-2360 proteins while the pPB1750-2360 and pMB1970-2360 (Gerbu) preparations reacted strongly with all four proteins. The Western blot reactivity of the pPB1750-2360 and pMB1970-2360 (Gerbu) preparations were equivalent to that of the CTB preparation, while reactivity of the pMB1970-2360 preparation was <10% that of the CTB prep. Despite equivalent ELISA reactivities only weak reactivity (approximately 1%) to the recombinant proteins were observed in PEG preps from two independent groups immunized with the pMB1750-2360 protein and one group immunized with the pMB1750-2360 preparation using Freund's adjuvant.

Affinity purification was utilized to determine if this difference in immunoreactivity by Western blot analysis reflects differing antibody titers. Fifty ml 2X PEG preparations from chickens immunized with either pMB1750-2360 or pMB1970-2360 protein were chromatographed on the pPB1750-2360 affinity column from b) above, as described. The yield of affinity purified antibody (% total protein in preparation) was equivalent to the yield obtained from a CTB PEG preparation in b) above. Thus, differences in Western reactivity reflect a qualitative difference in the antibody pools, rather than quantitative differences. These results demonstrate that certain recombinant proteins are more effective at generating high affinity antibodies (as assayed by Western blot hybridization).

iii) ***In Vivo* Neutralization Of Toxin B Using  
Antibodies Reactive To Recombinant  
Protein**

5 The *in vivo* hamster model [described in Examples 9 and 14(b)] was utilized to assess the neutralizing ability of antibodies raised against recombinant toxin B proteins. The results from three experiments are shown below in Tables 27-29.

10 The ability of each immunogen to neutralize toxin B *in vivo* has been compiled and is shown in Table 30. As predicted from the recombinant protein-CTB premix studies (Table 24) only antibodies to Interval 3 (1750-2366) and not the other regions of toxin B (*i.e.*, intervals 1-5) are protective. Unexpectedly, antibodies generated to  
15 INT-3 region expressed in pMAL vector (pMB1750-2360 and pMpB1750-2360) using Freund's adjuvant were non-neutralizing. This observation is reproducible, since no neutralization was observed in two independent immunizations with pMB1750-2360 and one immunization with pMpB1750-2360. The fact that 5X quantities of affinity  
20 purified toxin B repeat specific antibodies from pMB1750-2360 PEG preps cannot neutralize toxin B while 1X quantities of affinity purified anti-CTB antibodies can (Table 28) demonstrates that the differential ability of CTB antibodies to neutralize toxin B is due to qualitative rather than quantitative differences in these antibody  
25 preparations. Only when this region was expressed in an alternative vector (pPB1750-2360) or using an alternative adjuvant with the pMB1750-2360 protein were neutralizing antibodies generated. Importantly, antibodies raised using Freund's adjuvant to pPB1850-2360, which contains a fragment that is only 100 amino acids smaller than recombinant pPB1750-2360, are unable to neutralize toxin B *in vivo* (Table 27); note also that the same vector is used for both pPB1850-2360 and pPB1750-2360.

TABLE 27

*In Vivo* Neutralization Of Toxin B

Treatment Group <sup>a</sup>	Number Animals Alive <sup>b</sup>	Number Animals Dead <sup>b</sup>
Preimmune	0	5
CTB	5	0
INT1+2	0	5
INT 4+5	0	5
pMB1750-2360	0	5
pMB1970-2360	0	5
pPB1750-2360	5	0

<sup>a</sup> *C. difficile* toxin B (CTB) (at 5 µg/ml; 25 µg total; Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as a 4X antibody PEG prep.

<sup>b</sup> The numbers in each group represent numbers of hamsters dead or alive, 2 hours post IP administration of toxin/antibody mixture.

TABLE 28

*In Vivo* Neutralization Of Toxin B Using Affinity Purified Antibodies

Treatment Group <sup>a</sup>	Number Animals Alive <sup>b</sup>	Number Animals Dead <sup>b</sup>
Preimmune(1)	0	5
CTB(1)	5	0
pPB1750-2360(1)	5	0
1.5 mg anti-pMB1750-2360(2)	1	4
1.5 mg anti-pMB1970-2360(2)	0	5
300 µg anti-CTB(2)	5	0

<sup>a</sup> *C. difficile* toxin B (CTB) (at 5 µg/ml; 25 µg total; Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation, 1 ml of this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as either (1) 4X antibody PEG prep or (2) affinity purified antibody (on a pPB1750-2360 resin), either 1.5 mg/group (anti-pMB1750-2360 and anti-pMB1970-2360; used undiluted affinity purified antibody) or 350 µg/group (anti-CTB, repeat specific; used 1/5 diluted anti-CTB antibody).

<sup>b</sup> The numbers in each group represent numbers of hamsters dead or alive, 2 hr post-IP administration of toxin/antibody mixture.

TABLE 29

## Generation Of Neutralizing Antibodies Utilizing The Gerbu Adjuvant

Treatment Group <sup>a</sup>	Number Animals Alive <sup>b</sup>	Number Animals Dead <sup>b</sup>
Preimmune	0	5
CTB	5	0
pMB1970-2360	0	5
pMB1850-2360	0	5
pPB1850-2360	0	5
pMB1750-2360 (Gerbu adj)	5	0

<sup>a</sup> *C. difficile* toxin B (CTB) (Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as a 4X antibody PEG prep.

<sup>b</sup> The numbers in each group represent numbers of hamsters dead or alive, 2hrs post IP administration of toxin/antibody mixture.



TABLE 30

*In Vivo* Neutralization Of Toxin B

Immunogen	Adjuvant	Tested Preparation <sup>a</sup>	Antigen Utilized For AP	<i>In vivo</i> Neutralization <sup>b</sup>
Preimmune	NA <sup>1</sup>	PEG	NA	no
CTB (native)	Titermax	PEG	NA	yes
CTB (native)	Titermax	AP	pPB1750-2360	yes
CTB (native)	Titermax	AP	pPB1850-2360	yes
CTB (native)	Titermax	AP	pPB1750-1970	yes
CTB (native)	Titermax	AP	pPB1970-2360	yes
pMB1750-2360	Freunds	PEG	NA	no
pMB1750-2360	Freunds	AP	pPB1750-2360	no
pMB1750-2360	Gerbü	PEG	NA	yes
pMB1970-2360	Freunds	PEG	NA	no
pMB1970-2360	Freunds	AP	pPB1750-2360	no
pPB1750-2360	Freunds	PEG	NA	yes
pPB1850-2360	Freunds	PEG	NA	no
pMB1850-2360	Freunds	PEG	NA	no
INT 1+2	Freunds	PEG	NA	no
INT 4+5	Freunds	PEG	NA	no

<sup>a</sup> Either PEG preparation (PEG) or affinity purified antibodies (AP).

<sup>b</sup> 'Yes' denotes complete neutralization (0/5 dead) while 'no' denotes no neutralization (5/5 dead) of toxin B, 2 hours post-administration of mixture.

<sup>1</sup> 'NA' denotes not applicable.

The pPB1750-2360 antibody pool confers significant *in vivo* protection, equivalent to that obtained with the affinity purified CTB antibodies. This correlates with the observed high affinity of this antibody pool (relative to the pMB1750-2360 or pMB1970-2360 pools) as assayed by Western blot analysis (Figure 24). These results provide the first demonstration that *in vivo* neutralizing antibodies can be induced using recombinant toxin B protein as immunogen.

The failure of high concentrations of antibodies raised against the pMB1750-2360 protein (using Freund's adjuvant) to neutralize, while the use of Gerbu adjuvant and pMB1750-2360 protein generates a neutralizing response, demonstrates that conformation or presentation of this protein is essential for the induction of neutralizing antibodies. These results are consistent with the observation that the neutralizing antibodies produced when native CTB is used as an immunogen appear to recognize conformational epitopes [see section b) above]. This is the first demonstration that the conformation or presentation of recombinant toxin B protein is essential to generate high titers of neutralizing antibodies.

## EXAMPLE 20

### Determination Of Quantitative And Qualitative Differences Between pMB1750-2360, pMB1750-2360 (Gerbu) Or pPB1750-2360 IgY Polyclonal Antibody Preparations

In Example 19, it was demonstrated that toxin B neutralizing antibodies could be generated using specific recombinant toxin B proteins (pPB1750-2360) or specific adjuvants. Antibodies raised against pMB1750-2360 were capable of neutralizing the enterotoxin effect of toxin B when the recombinant protein was used to immunize hens in conjunction with the Gerbu adjuvant, but not when Freund's adjuvant was used. To determine the basis for these antigen and adjuvant restrictions, toxin B-specific antibodies present in the neutralizing and non-neutralizing PEG preparations were isolated by affinity chromatography and tested for qualitative or quantitative differences. The example involved a) purification of anti-toxin B specific antibodies from pMB1750-2360 and pPB1750-2360 PEG preparations and b) *in vivo* neutralization of toxin B using the affinity purified antibody.

**a) Purification Of specific Antibodies From  
pMB1750-2360 And pPB1750-2360 PEG Preparations**

To purify and determine the concentration of specific antibodies (expressed as the percent of total antibody) within the pPB1750-2360 (Freunds and Gerbu) and  
5 pPB1750-2360 PEG preparations, defined quantities of these antibody preparations were chromatographed on an affinity column containing the entire toxin B repeat region (pPB1750-2360). The amount of affinity purified antibody was then quantified.

An affinity column containing the recombinant toxin B repeat protein, pPB1750-2360, was made as follows. Four ml of PBS-washed Actigel resin  
10 (Sterogene) was coupled with 5 mg of pPB1750-2360 affinity purified protein (dialyzed into PBS; estimated to be greater than 95% full length fusion protein) in a 15 ml tube (Falcon) containing 1/10 final volume Ald-coupling solution (1M sodium cyanoborohydride). Aliquots of the supernatant from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels.  
15 Based on protein band intensities, greater than 95% (approximately 5 mg) of recombinant protein was coupled to the resin. The coupled resin was poured into a 10 ml column (BioRad), washed extensively with PBS, pre-eluted with 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal) and re-equilibrated in PBS and stored at 4°C.

20 Aliquots of pMB1750-2360, pMB1750-2360 (Gerbu) or pPB1750-2360 IgY polyclonal antibody preparations (PEG preps) were affinity purified on the above column as follows. The column was attached to an UV monitor (ISCO), and washed with PBS. Forty ml aliquots of 2X PEG preps (filter sterilized using a 0.45  $\mu$  filter and quantified by OD<sub>280</sub> before chromatography) was applied. The column was  
25 washed with PBS until the baseline was re-established (the column flow-through was saved), washed with BBSTween to elute nonspecifically binding antibodies and re-equilibrated with PBS. Bound antibody was eluted from the column in 4M guanidine-HCl (in 10 mM Tris-HCL, pH 8.0, 0.005% thimerosal) and the entire elution peak collected in a 15 ml tube (Falcon). The column was re-equilibrated, and the column  
30 eluate re-chromatographed as described above. The antibody preparations were

quantified by UV absorbance (the elution buffer was used to zero the spectrophotometer). Approximately 10 fold higher concentrations of total purified antibody was obtained upon elution of the first chromatography pass relative to the second pass. The low yield from the second chromatography pass indicated that most of the specific antibodies were removed by the first round of chromatography.

Pools of affinity purified specific antibodies were prepared by dialysis of the column elutes after the first column chromatography pass for the pMB1750-2360, pMB1750-2360 (Gerbu) or pPB1750-2360 IgY polyclonal antibody preparations. The elutes were collected on ice and immediately dialyzed against a 100-fold volume of PBS at 4°C for 2 hrs. The samples were then dialyzed against 3 changes of a 65-fold volume of PBS at 4°C. Dialysis was performed for a minimum of 8 hrs per change of PBS. The dialyzed samples were collected, centrifuged to remove insoluble debris, quantified by OD<sub>280</sub>, and stored at 4°C.

The percentage of toxin B repeat-specific antibodies present in each preparation was determined using the quantifications of antibody yields from the first column pass (amount of specific antibody recovered after first pass/total protein loaded). The yield of repeat-specific affinity purified antibody (expressed as the percent of total protein in the preparation) in: 1) the pMB1750-2360 PEG prep was approximately 0.5%, 2) the pMB1750-2360 (Gerbu) prep was approximately 2.3%, and 3) the pPB1750-2360 prep was approximately 0.4%. Purification of a CTB IgY polyclonal antibody preparation on the same column demonstrated that the concentration of toxin B repeat specific antibodies in the CTB preparation was 0.35%.

These results demonstrate that 1) the use of Gerbu adjuvant enhanced the titer of specific antibody produced against the pMB1750-2360 protein 5-fold relative to immunization using Freund's adjuvant, and 2) the differences seen in the *in vivo* neutralization ability of the pMB1750-2360 (not neutralizing) and pPB1750-2360 (neutralizing) and CTB (neutralizing) PEG preps seen in Example 19 was not due to differences in the titers of repeat-specific antibodies in the three preparations because the titer of repeat-specific antibody was similar for all three preps; therefore the differing ability of the three antibody preparations to neutralize toxin B must reflect

qualitative differences in the induced toxin B repeat-specific antibodies. To confirm that qualitative differences exist between antibodies raised in hens immunized with different recombinant proteins and/or different adjuvants, the same amount of affinity purified anti-toxin B repeat (aa 1870-2360 of toxin B) antibodies from the different preparations was administered to hamsters using the *in vivo* hamster model as described below.

**b) *In vivo* Neutralization Of Toxin B Using Affinity Purified Antibody**

The *in vivo* hamster model was utilized to assess the neutralizing ability of the affinity purified antibodies raised against recombinant toxin B proteins purified in (a) above. As well, a 4X IgY PEG preparation from a second independent immunization utilizing the pPB1750-2360 antigen with Freund's adjuvant was tested for *in vivo* neutralization. The results are shown in Table 31.

The results shown in Table 31 demonstrate that:

- 1) as shown in Example 19 and reproduced here, 1.5 mg of affinity purified antibody from pMB1750-2360 immunized hens using Freund's adjuvant does not neutralize toxin B *in vivo*. However, 300 µg of affinity purified antibody from similarly immunized hens utilizing Gerbu adjuvant demonstrated complete neutralization of toxin B *in vivo*. This demonstrates that Gerbu adjuvant, in addition to enhancing the titer of antibodies reactive to the pMB1750-2360 antigen relative to Freund's adjuvant (demonstrated in (a) above), also enhances the yield of neutralizing antibodies to this antigen, greater than 5 fold.
- 2) Complete *in vivo* neutralization of toxin B was observed with 1.5 mg of affinity purified antibody from hens immunized with pPB1750-2360 antigen, but not with pMB1750-2360 antigen, when Freund's adjuvant was used. This demonstrates, using standardized toxin B repeat-specific antibody concentrations, that neutralizing antibodies were induced when

pPB1750-2360 but not pMB1750-2360 was used as the antigen with Freund's adjuvant.

- 3) Complete *in vivo* neutralization was observed with 300 µg of pMB1750-2360 (Gerbu) antibody, but not with 300 µg of pPB1750-2360 (Freund's) antibody. Thus the pMB1750-2360 (Gerbu) antibody has a higher titer of neutralizing antibodies than the pPB1750-2360 (Freund's) antibody.
- 4) Complete neutralization of toxin B was observed using 300 µg of CTB antibody [affinity purified (AP)] but not 100 µg CTB antibody (AP or PEG prep). This demonstrates that greater than 100 µg of toxin B repeat-specific antibody (anti-CTB) is necessary to neutralize 25 µg toxin B *in vivo* in this assay, and that affinity purified antibodies specific to the toxin B repeat interval neutralize toxin B as effectively as the PEP prep of IgY raised against the entire CTB protein (shown in this assay).
- 5) As was observed with the initial pPB1750-2360 (IgY) PEG preparation (Example 19), complete neutralization was observed with a IgY PEG preparation isolated from a second independent group of pPB1750-2360 (Freund's) immunized hens. This demonstrates that neutralizing antibodies are reproducibly produced when hens are immunized with pPB1750-2360 protein utilizing Freund's adjuvant.

TABLE 31

*In vivo* Neutralization Of Toxin B Using Affinity Purified Antibodies

Treatment Group <sup>a</sup>	Number Animals Alive <sup>b</sup>	Number Animals Dead <sup>b</sup>
Preimmune <sup>1</sup>	0	5
CTB (300 µg) <sup>2</sup>	5	0
CTB (100 µg) <sup>2</sup>	1	4
pMB1750-2360 (G) (5 mg) <sup>2</sup>	5	0
pMB1750-2360 (G) (1.5 mg) <sup>2</sup>	5	0
pMB1750-2360 (G) (300 µg) <sup>2</sup>	5	0
pMB1750-2360 (F) (1.5 mg) <sup>2</sup>	0	5
pPB1750-2360 (F) (1.5 mg) <sup>2</sup>	5	0
pPB1750-2360 (F) (300 µg) <sup>2</sup>	1	4
CTB (100 µg) <sup>3</sup>	2	3
pPB1750-2360 (F) (500 µg) <sup>1</sup>	5	0

<sup>a</sup> *C. difficile* toxin B (CTB) (Tech Lab) at lethal concentration to hamsters (25 µg) was added to the antibody (amount of specific antibody is indicated) and incubated for one hour at 37°C. After incubation, this mixture was injected IP into hamsters (1/5 total mix injected per hamster). Each treatment group received toxin premixed with antibody raised against the indicated protein (G=gerbu adjuvant, F=Freunds adjuvant). <sup>1</sup> indicates the antibody was a 4X IgY PEG prep; <sup>2</sup> indicates the antibody was affinity purified on a pPB1850-2360 resin; and <sup>3</sup> indicates that the antibody was a 1X IgY PEG prep.

<sup>b</sup> The numbers in each group represent numbers of hamsters dead or alive, 2 hrs post IP administration of toxin/antibody mixture.

### EXAMPLE 21

#### Diagnostic Enzyme Immunoassays For *C. difficile* Toxins A And B

The ability of the recombinant toxin proteins and antibodies raised against these recombinant proteins (described in the above examples) to form the basis of diagnostic assays for the detection of clostridial toxin in a sample was examined. Two immunoassay formats were tested to quantitatively detect *C. difficile* toxin A and toxin B from a biological specimen. The first format involved a competitive assay in which





into the first row of wells in the microtiter plate, and 50  $\mu$ l aliquots were removed and diluted serially down the plate in duplicate. An equal volume of affinity purified anti-recombinant toxin antibodies [1 ng/well of anti-pMA1870-2680 antibody was used for the detection of toxin A; 0.5 ng/well of anti-pMB1750-2360(Gerbu) was used for the detection of toxin B] were added to appropriate wells, and the plates were incubated at room temperature for 2 hours with gentle agitation. Wells serving as negative control contained antibody but no native toxin to compete for binding.

Unbound toxin and antibody were removed by washing the plates 3 to 5 times with PBS containing 0.05% Tween-20. Following the wash step, 100  $\mu$ l of rabbit anti-chicken IgG antibody conjugated to alkaline phosphatase (Sigma) was added to each well and the plates were incubated for 2 hours at room temperature. The plates were then washed as before to remove unbound secondary antibody. Freshly prepared alkaline phosphatase substrate (1 mg/ml p-nitrophenyl phosphate (Sigma) in 50 mM  $\text{Na}_2\text{CO}_3$ , pH 9.5; 10 mM  $\text{MgCl}_2$ ) was added to each well. Once sufficient color developed, the plates were read on a Dynatech MR700 microtiter plate reader using a 410 nm filter.

The results are summarized in Tables 32 and 33. For the results shown in Table 32, the wells were coated with recombinant toxin A protein (pMA1870-2680). The amount of native toxin A added (present as an addition to solubilized hamster stool) to a given well is indicated (0 to 200 ng). Antibody raised against the recombinant toxin A protein, pMA1870-2680, was affinity purified on the an affinity column containing pPA1870-2680 (described in Example 20). As shown in Table 32, the recombinant toxin A protein and affinity-purified antitoxin can be used for the basis of a competitive immunoassay for the detection of toxin A in biological samples.

Similar results were obtained using the recombinant toxin B, pPB1750-2360, and antibodies raised against pMB1750-2360(Gerbu). For the results shown in Table 33, the wells were coated with recombinant toxin B protein (pPB1750-2360). The amount of native toxin B added (present as an addition to solubilized hamster stool) to a given well is indicated (0 to 200 ng). Antibody raised against the recombinant toxin B protein, pMB1750-2360(Gerbu), was affinity purified on the an

affinity column containing pPB1850-2360 (described in Example 20). As shown in Table 33, the recombinant toxin B protein and affinity-purified antitoxin can be used for the basis of a competitive immunoassay for the detection of toxin B in biological samples.

5 In this competition assay, the reduction is considered significant over the background levels at all points; therefore the assay can be used to detect samples containing less than 12.5 ng toxin A/well and as little as 50-100 ng toxin B/well.

**TABLE 32**

Competitive Inhibition Of Anti-*C. difficile* Toxin A By Native Toxin A

10	ng Toxin A/Well	OD <sub>410</sub> Readout
	200	0.176
	100	0.253
	50	0.240
	25	0.259
15	12.5	0.309
	6.25	0.367
	3.125	0.417
	0	0.590

TABLE 33

Competitive Inhibition Of Anti-*C. difficile* Toxin B By Native Toxin B

ng Toxin B/Well	OD <sub>410</sub> Readout
200	0.392
100	0.566
50	0.607
25	0.778
12.5	0.970
6.25	0.902
3.125	1.040
0	1.055

These competitive inhibition assays demonstrate that native *C. difficile* toxins and recombinant *C. difficile* toxin proteins can compete for binding to antibodies raised against recombinant *C. difficile* toxins demonstrating that these anti-recombinant toxin antibodies provide effective diagnostic reagents.

**b) Sandwich Immunoassay For The Detection Of  
*C. difficile* Toxin**

Affinity-purified antibodies against recombinant toxin A or toxin B were immobilized to 96 well microtiter plates as follows. The wells were passively coated overnight at 4°C with affinity purified antibodies raised against either pMA1870-2680 (toxin A) or pMB1750-2360(Gerbu) (toxin B). The antibodies were affinity purified as described in Example 20. The antibodies were used at a concentration of 1 µg/ml and 100 µl was added to each microtiter well. The wells were then blocked with 200 µl of 0.5% BSA in PBS for 2 hours at room temperature and the blocking solution was then decanted. Stool samples from healthy Syrian hamsters were resuspended in PBS, pH 7.4 (2 ml PBS/stool pellet was used to resuspend the pellets and the sample was centrifuged as described above). The stool suspension was then spiked with native *C. difficile* toxin A or B (Tech Lab) at 4 µg/ml. The stool suspensions

containing toxin (either toxin A or toxin B) were then serially diluted two-fold in stool suspension without toxin and 50  $\mu$ l was added in duplicate to the coated microtiter wells. Wells containing stool suspension without toxin served as the negative control.

The plates were incubated for 2 hours at room temperature and then were washed three times with PBS. One hundred  $\mu$ l of either goat anti-native toxin A or goat anti-native toxin B (Tech Lab) diluted 1:1000 in PBS containing 1% BSA and 0.05% Tween 20 was added to each well. The plates were incubated for another 2 hours at room temperature. The plates were then washed as before and 100  $\mu$ l of alkaline phosphatase-conjugated rabbit anti-goat IgG (Cappel, Durham, N.C.) was added at a dilution of 1:1000. The plates were incubated for another 2 hours at room temperature. The plates were washed as before then developed by the addition of 100  $\mu$ l/well of a substrate solution containing 1 mg/ml p-nitrophenyl phosphate (Sigma) in 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5; 10 mM MgCl<sub>2</sub>. The absorbance of each well was measured using a plate reader (Dynatech) at 410 nm. The assay results are shown in Tables 34 and 35.

TABLE 34

*C. difficile* Toxin A Detection In Stool Using Affinity-Purified Antibodies Against Toxin A

ng Toxin A/Well	OD <sub>410</sub> Readout
200	0.9
100	0.8
50	0.73
25	0.71
12.5	0.59
6.25	0.421
0	0

TABLE 35

*C. difficile* Toxin B Detection In Stool Using Affinity-Purified Antibodies Against Toxin B

ng Toxin B/Well	OD <sub>410</sub> Readout
200	1.2
100	0.973
50	0.887
25	0.846
12.5	0.651
6.25	0.431
0	0.004

The results shown in Tables 34 and 35 show that antibodies raised against recombinant toxin A and toxin B fragments can be used to detect the presence of *C. difficile* toxin in stool samples. These antibodies form the basis for a sensitive sandwich immunoassay which is capable of detecting as little as 6.25 ng of either toxin A or B in a 50  $\mu$ l stool sample. As shown above in Tables 34 and 35, the background for this sandwich immunoassay is extremely low; therefore, the sensitivity of this assay is much lower than 6.25 ng toxin/well. It is likely that toxin levels of 0.5 to 1.0 pg/well could be detected by this assay.

The results shown above in Tables 32-35 demonstrate clear utility of the recombinant reagents in *C. difficile* toxin detection systems.

## EXAMPLE 22

### Construction And Expression Of *C. botulinum* C Fragment Fusion Proteins

The *C. botulinum* type A neurotoxin gene has been cloned and sequenced [Thompson, *et al.*, Eur. J. Biochem. 189:73 (1990)]. The nucleotide sequence of the toxin gene is available from the EMBL/GenBank sequence data banks under the accession number X52066; the nucleotide sequence of the coding region is listed in SEQ ID NO:27. The amino acid sequence of the *C. botulinum* type A neurotoxin is

listed in SEQ ID NO:28. The type A neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H<sub>C</sub> domain.

5           Previous attempts by others to express polypeptides comprising the C fragment of *C. botulinum* type A toxin as a native polypeptide (e.g., not as a fusion protein) in *E. coli* have been unsuccessful [H.F. LaPenotiere, *et al.* in *Botulinum and Tetanus Neurotoxins*, DasGupta, Ed., Plenum Press, New York (1993), pp. 463-466]. Expression of the C fragment as a fusion with the *E. coli* MBP was reported to result  
10           in the production of insoluble protein (H.F. LaPenotiere, *et al.*, *supra*).

          In order to produce soluble recombinant C fragment proteins in *E. coli*, fusion proteins comprising a synthetic C fragment gene derived from the *C. botulinum* type A toxin and either a portion of the *C. difficile* toxin protein or the MBP were constructed. This example involved a) the construction of plasmids encoding C  
15           fragment fusion proteins and b) expression of *C. botulinum* C fragment fusion proteins in *E. coli*.

**a)       Construction Of Plasmids Encoding C Fragment  
          Fusion Proteins**

          In Example 11, it was demonstrated that the *C. difficile* toxin A repeat domain  
20           can be efficiently expressed and purified in *E. coli* as either native (expressed in the pET 23a vector in clone pPA1870-2680) or fusion (expressed in the pMALc vector as a fusion with the *E. coli* MBP in clone pMA1870-2680) proteins. Fusion proteins comprising a fusion between the MBP, portions of the *C. difficile* toxin A repeat domain (shown to be expressed as a soluble fusion protein) and the C fragment of the  
25           *C. botulinum* type A toxin were constructed. A fusion protein comprising the C fragment of the *C. botulinum* type A toxin and the MBP was also constructed.

          Figure 25 provides a schematic representation of the botulinal fusion proteins along with the donor constructs containing the *C. difficile* toxin A sequences or *C.*

*botulinum* C fragment sequences which were used to generate the botulinal fusion proteins. In Figure 25, the solid boxes represent *C. difficile* toxin A gene sequences, the open boxes represent *C. botulinum* C fragment sequences and the solid black ovals represent the *E. coli* MBP. When the name for a restriction enzyme appears inside  
5 parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at the cloning junction.

In Figure 25, a restriction map of the pMA1870-2680 and pPA1100-2680 constructs (described in Example 11) which contain sequences derived from the *C.  
10 difficile* toxin A repeat domain are shown; these constructs were used as the source of *C. difficile* toxin A gene sequences for the construction of plasmids encoding fusions between the *C. botulinum* C fragment gene and the *C. difficile* toxin A gene. The pMA1870-2680 expression construct expresses high levels of soluble, intact fusion protein (20 mg/liter culture) which can be affinity purified on an amylose column  
15 (purification described in Example 11d).

The pAlterBot construct (Figure 25) was used as the source of *C. botulinum* C fragment gene sequences for the botulinal fusion proteins. pAlterBot was obtained from J. Middlebrook and R. Lemley at the U.S. Department of Defense. pAlterBot contains a synthetic *C. botulinum* C fragment inserted in to the pALTER-1® vector  
20 (Promega). This synthetic C fragment gene encodes the same amino acids as does the naturally occurring C fragment gene. The naturally occurring C fragment sequences, like most clostridial genes, are extremely A/T rich (Thompson *et al.*, *supra*). This high A/T content creates expression difficulties in *E. coli* and yeast due to altered codon usage frequency and fortuitous polyadenylation sites, respectively. In order to  
25 improve the expression of C fragment proteins in *E. coli*, a synthetic version of the gene was created in which the non-preferred codons were replaced with preferred codons.

The nucleotide sequence of the *C. botulinum* C fragment gene sequences contained within pAlterBot is listed in SEQ ID NO:22. The first six nucleotides

(ATGGCT) encode a methionine and alanine residue, respectively. These two amino acids result from the insertion of the *C. botulinum* C fragment sequences into the pALTER® vector and provide the initiator methionine residue. The amino acid sequence of the *C. botulinum* C fragment encoded by the sequences contained within  
5 pAlterBot is listed in SEQ ID NO:23. The first two amino acids (Met Ala) are encoded by vector-derived sequences. From the third amino acid residue onward (Arg), the amino acid sequence is identical to that found in the *C. botulinum* type A toxin gene.

The pMA1870-2680, pPA1100-2680 and pAlterBot constructs were used as  
10 progenitor plasmids to make expression constructs in which fragments of the *C. difficile* toxin A repeat domain were expressed as genetic fusions with the *C. botulinum* C fragment gene using the pMAL-c expression vector (New England BioLabs). The pMAL-c expression vector generates fusion proteins which contain the MBP at the amino-terminal end of the protein. A construct, pMBot, in which the *C.*  
15 *botulinum* C fragment gene was expressed as a fusion with only the MBP was constructed (Figure 25). Fusion protein expression was induced from *E. coli* strains harboring the above plasmids, and induced protein was affinity purified on an amylose resin column.

#### i) Construction Of pBlueBot

20 In order to facilitate the cloning of the *C. botulinum* C fragment gene sequences into a number of desired constructs, the botulinal gene sequences were removed from pAlterBot and were inserted into the pBluescript plasmid (Stratagene) to generate pBlueBot (Figure 25). pBlueBot was constructed as follows. Bacteria containing the pAlterBot plasmid were grown in medium containing tetracycline and  
25 plasmid DNA was isolated using the QIAprep-spin Plasmid Kit (Qiagen). One microgram of pAlterBot DNA was digested with *Nco*I and the resulting 3' recessed sticky end was made blunt using the Klenow fragment of DNA polymerase I (here after the Klenow fragment). The pAlterBot DNA was then digested with *Hind*III to



release the botulinal gene sequences (the Bot insert) as a blunt (filled *NcoI* site)-  
*HindIII* fragment. pBluescript vector DNA was prepared by digesting 200 ng of  
pBluescript DNA with *SmaI* and *HindIII*. The digestion products from both plasmids  
were resolved on an agarose gel. The appropriate fragments were removed from the  
5 gel, mixed and purified utilizing the Prep-a-Gene kit (BioRad). The eluted DNA was  
then ligated using T4 DNA ligase and used to transform competent DH5 $\alpha$  cells  
(Gibco-BRL). Host cells were made competent for transformation using the calcium  
chloride protocol of Sambrook *et al.*, *supra* at 1.82-1.83. Recombinant clones were  
isolated and confirmed by restriction digestion using standard recombinant molecular  
10 biology techniques (Sambrook *et al.*, *supra*). The resultant clone, pBlueBot, contains  
several useful unique restriction sites flanking the Bot insert (*i.e.*, the *C. botulinum* C  
fragment sequences derived from pAlterBot) as shown in Figure 25.

ii)      **Construction Of *C. difficile* /  
*C. botulinum* / MBP Fusion Proteins**

15            Constructs encoding fusions between the *C. difficile* toxin A gene and the  
*C. botulinum* C fragment gene and the MBP were made utilizing the same recombinant  
DNA methodology outlined above; these fusion proteins contained varying amounts of  
the *C. difficile* toxin A repeat domain.

20            The pMABot clone contains a 2.4 kb insert derived from the *C. difficile* toxin  
A gene fused to the Bot insert (*i.e.*, the *C. botulinum* C fragment sequences derived  
from pAlterBot). pMABot (Figure 25) was constructed by mixing gel-purified DNA  
from *NotI/HindIII* digested pBlueBot (the 1.2 kb Bot fragment), *SpeI/NotI* digested  
pPA1100-2680 (the 2.4 kb *C. difficile* toxin A repeat fragment) and *XbaI/HindIII*  
25 digested pMAL-c vector. Recombinant clones were isolated, confirmed by restriction  
digestion and purified using the QIAprep-spin Plasmid Kit (Qiagen). This clone  
expresses the toxin A repeats and the botulinal C fragment protein sequences as an in-  
frame fusion with the MBP.

The pMCABot construct contains a 1.0 kb insert derived from the *C. difficile* toxin A gene fused to the Bot insert (*i.e.*, the *C. botulinum* C fragment sequences derived from pAlterBot). pMCABot was constructed by digesting the pMABot clone with *EcoRI* to remove the 5' end of the *C. difficile* toxin A repeat (see Figure 25, the pMAL-c vector contains a *EcoRI* site 5' to the *C. difficile* insert in the pMABot clone). The restriction sites were filled and religated together after gel purification. The resultant clone (pMCABot, Figure 25) generated an in-frame fusion between the MBP and the remaining 3' portion of the *C. difficile* toxin A repeat domain fused to the Bot gene.

The pMNABot clone contains the 1 kb *SpeI/EcoRI* (filled) fragment from the *C. difficile* toxin A repeat domain (derived from clone pPA1100-2680) and the 1.2 kb *C. botulinum* C fragment gene as a *NcoI* (filled)/*HindIII* fragment (derived from pAlterBot). These two fragments were inserted into the pMAL-c vector digested with *XbaI/HindIII*. The two insert fragments were generated by digestion of the appropriate plasmid with *EcoRI* (pPA1100-2680) or *NcoI* (pAlterBot) followed by treatment with the Klenow fragment. After treatment with the Klenow fragment, the plasmids were digested with the second enzyme (either *SpeI* or *HindIII*). All three fragments were gel purified, mixed and Prep-a-Gene purified prior to ligation. Following ligation and transformation, putative recombinants were analyzed by restriction analysis; the *EcoRI* site was found to be regenerated at the fusion junction, as was predicted for a fusion between the filled *EcoRI* and *NcoI* sites.

A construct encoding a fusion protein between the botulinal C fragment gene and the MBP gene was constructed (*i.e.*, this fusion lacks any *C. difficile* toxin A gene sequences) and termed pMBot. The pMBot construct was made by removal of the *C. difficile* toxin A sequences from the pMABot construct and fusing the C fragment gene sequences to the MBP. This was accomplished by digestion of pMABot DNA with *StuI* (located in the pMALc polylinker 5' to the *XbaI* site) and *XbaI* (located 3' to the *NotI* site at the toxA-Bot fusion junction), filling in the *XbaI* site using the Klenow fragment, gel purifying the desired restriction fragment, and ligating the blunt ends to

circularize the plasmid. Following ligation and transformation, putative recombinants were analyzed by restriction mapping of the Bot insert (*i.e.*, the *C. botulinum* C fragment sequences).

**b) Expression Of *C. botulinum* C Fragment Fusion  
Proteins In *E. coli***

Large scale (1 liter) cultures of the pMAL-c vector, and each recombinant construct described above in (a) were grown, induced, and soluble protein fractions were isolated as described in Example 18. The soluble protein extracts were chromatographed on amylose affinity columns to isolate recombinant fusion protein. The purified recombinant fusion proteins were analyzed by running samples on SDS-PAGE gels followed by Coomassie staining and by Western blot analysis as described [Williams *et al.*, (1994) *supra*]. In brief, extracts were prepared and chromatographed in column buffer (10 mM NaPO<sub>4</sub>, 0.5 M NaCl, 10 mM β-mercaptoethanol, pH 7.2) over an amylose resin (New England Biolabs) column, and eluted with column buffer containing 10 mM maltose as described [Williams, *et al.* (1994), *supra*]. An SDS-PAGE gel containing the purified protein samples stained with Coomassie blue is shown in Figure 26.

In Figure 26, the following samples were loaded. Lanes 1-6 contain protein purified from *E. coli* containing the pMAL-c, pPA1870-2680, pMABot, pMNABot, pMCABot and pMBot plasmids, respectively. Lane 7 contains broad range molecular weight protein markers (BioRad).

The protein samples were prepared for electrophoresis by mixing 5 μl of eluted protein with 5 μl of 2X SDS-PAGE sample buffer (0.125 mM Tris-HCl, pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 0.025% bromophenol blue; β-mercaptoethanol is added to 5% before use). The samples were heated to 95°C for 5 min, then cooled and loaded on a 7.5% agarose SDS-PAGE gel. Broad range molecular weight protein markers were also loaded to allow estimation of the MW of identified fusion proteins.

After electrophoresis, protein was detected generally by staining the gel with Coomassie blue.

In all cases the yields were in excess of 20 mg fusion protein per liter culture (see Table 36) and, with the exception of the pMCABot protein, a high percentage (i.e., greater than 20-50% of total eluted protein) of the eluted fusion protein was of a MW predicted for the full length fusion protein (Figure 26). It was estimated (by visual inspection) that less than 10% of the pMCABot fusion protein was expressed as the full length fusion protein.

TABLE 36

Yield Of Affinity Purified *C. botulinum* C Fragment / MBP Fusion Proteins

Construct	Yield (mg/liter of Culture)	Percentage Of Total Soluble Protein
pMABot	24	5.0
pMCABot	34	5.0
pMNABot	40	5.5
pMBot	22	5.0
pMA1870-2680	40	4.8

These results demonstrate that high level expression of intact *C. botulinum* C fragment/*C. difficile* toxin A fusion proteins in *E. coli* is feasible using the pMAL-c expression system. These results are in contrast to those reported by H. F. LaPenotiere, *et al.* (1993), *supra*. In addition, these results show that it is not necessary to fuse the botulinal C fragment gene to the *C. difficile* toxin A gene in order to produce a soluble fusion protein using the pMAL-c system in *E. coli*.

In order to determine whether the above-described botulinal fusion proteins were recognized by anti-*C. botulinum* toxin A antibodies, Western blots were performed. Samples containing affinity-purified proteins from *E. coli* containing the pMABot, pMCABot, pMNABot, pMBot, pMA1870-2680 or pMALc plasmids were analyzed. SDS-PAGE gels (7.5% acrylamide) were loaded with protein samples purified from each expression construct. After electrophoresis, the gels were blotted

and protein transfer was confirmed by Ponceau S staining (as described in Example 12b).

Following protein transfer, the blots were blocked by incubation for 1 hr at 20°C in blocking buffer [PBST (PBS containing 0.1% Tween 20 and 5% dry milk)].

5 The blots were then incubated in 10 ml of a solution containing the primary antibody; this solution comprised a 1/500 dilution of an anti-*C. botulinum* toxin A IgY PEG prep (described in Example 3) in blocking buffer. The blots were incubated for 1 hr at room temperature in the presence of the primary antibody. The blots were washed and developed using a rabbit anti-chicken alkaline phosphatase conjugate (Boehringer

10 Mannheim) as the secondary antibody as follows. The rabbit anti-chicken antibody was diluted to 1 µg/ml in blocking buffer (10 ml final volume per blot) and the blots were incubated at room temperature for 1 hour in the presence of the secondary antibody. The blots were then washed successively with PBST, BBS-Tween and 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5. The blots were then developed in freshly-prepared alkaline phosphatase

15 substrate buffer (100 µg/ml nitro blue tetrazolium, 50 µg/ml 5-bromo-chloro-indolylphosphate, 5 mM MgCl<sub>2</sub> in 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5). Development was stopped by flooding the blots with distilled water and the blots were air dried.

This Western blot analysis detected anti-*C. botulinum* toxin reactive proteins in the pMABot, pMCABot, pMNABot and pMBot protein samples (corresponding to the

20 predicted full length proteins identified above by Coomassie staining in Figure 26), but not in the pMA1100-2680 or pMALc protein samples.

These results demonstrate that the relevant fusion proteins purified on an amylose resin as described above in section a) contained immunoreactive *C botulinum* C fragment protein as predicted.

### EXAMPLE 23

#### Generation Of Neutralizing Antibodies By Nasal Administration Of pMBot Protein

5 The ability of the recombinant botulinal toxin proteins produced in Example 22 to stimulate a systemic immune response against botulinal toxin epitopes was assessed. This example involved: a) the evaluation of the induction of serum IgG titers produced by nasal or oral administration of botulinal toxin-containing *C. difficile* toxin A fusion proteins and b) the *in vivo* neutralization of *C. botulinum* type A neurotoxin by anti- recombinant *C. botulinum* C fragment antibodies.

10           a)   **Evaluation Of The Induction Of Serum IgG Titers  
Produced By Nasal Or Oral Administration Of  
Botulinal Toxin-Containing *C. difficile* Toxin A Fusion  
Proteins**

15 Six groups containing five 6 week old CF female rats (Charles River) per group were immunized nasally or orally with one of the following three combinations using protein prepared in Example 22: (1) 250 µg pMBot protein per rat (nasal and oral); 2) 250 µg pMABot protein per rat (nasal and oral); 3) 125 µg pMBot admixed with 125 µg pMA1870-2680 per rat (nasal and oral). A second set of 5 groups containing 3 CF female rats/group were immunized nasally or orally with one of the following combinations (4) 250 µg pMNABot protein per rat (nasal and oral) or 5) 250 µg pMAL-c protein per rat (nasal and oral).

20 The fusion proteins were prepared for immunization as follows. The proteins (in column buffer containing 10 mM maltose) were diluted in 0.1 M carbonate buffer, pH 9.5 and administered orally or nasally in a 200 µl volume. The rats were lightly sedated with ether prior to administration. The oral dosing was accomplished using a 25 20 gauge feeding needle. The nasal dosing was performed using a P-200 micro-pipettor (Gilson). The rats were boosted 14 days after the primary immunization using the techniques described above and were bled 7 days later. Rats from each group were

lightly etherized and bled from the tail. The blood was allowed to clot at 37°C for 1 hr and the serum was collected.

The serum from individual rats was analyzed using an ELISA to determine the anti-*C. botulinum* type A toxin IgG serum titer. The ELISA protocol used is a modification of that described in Example 13c. Briefly, 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were coated with *C. botulinum* type A toxoid (prepared as described in Example 3a) by placing 100 µl volumes of *C. botulinum* type A toxoid at 2.5 µg/ml in PBS containing 0.005% thimerosal in each well and incubating overnight at 4°C. The next morning, the coating suspensions were decanted and all wells were washed three times using PBS.

In order to block non-specific binding sites, 100 µl of blocking solution [0.5% BSA in PBS] was then added to each well and the plates were incubated for 1 hr at 37°C. The blocking solution was decanted and duplicate samples of 150 µl of diluted rat serum added to the first well of a dilution series. The initial testing serum dilution was 1:30 in blocking solution containing 0.5% Tween 20 followed by 5-fold dilutions into this solution. This was accomplished by serially transferring 30 µl aliquots to 120 µl blocking solution containing 0.5% Tween 20, mixing, and repeating the dilution into a fresh well. After the final dilution, 30 µl was removed from the well such that all wells contained 120 µl final volume. A total of 3 such dilutions were performed (4 wells total). The plates were incubated 1 hr at 37°C. Following this incubation, the serially diluted samples were decanted and the wells were washed six times using PBS containing 0.5% Tween 20 (PBST). To each well, 100 µl of a rabbit anti-Rat IgG alkaline phosphatase (Sigma) diluted (1/1000) in blocking buffer containing 0.5% Tween 20 was added and the plate was incubated for 1 hr at 37°C. The conjugate solutions were decanted and the plates were washed as described above, substituting 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5 for the PBST in the final wash. The plates were developed by the addition of 100 µl of a solution containing 1 mg/ml para-nitro phenyl phosphate (Sigma) dissolved in 50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH 9.5 to each well, and incubating the plates at room temperature in the dark for 5-45 min. The absorbency of each well was measured at 410 nm using a Dynatech MR 700 plate reader. The

results are summarized in Tables 37 and 38 and represent mean serum reactivities of individual mice.

TABLE 37

Determination Of Anti-*C. botulinum* Type A Toxin Serum IgG Titers  
Following Immunization With *C. botulinum* C Fragment-Containing Fusion Proteins

Route of Immunization		Nasal			Oral		
Immunogen	PRE- IMMUNE	pMBot	pMBot & pMA1870- 2680	pMABot	pMBot	pMBot& pMA1870- 2680	pMABot
Dilution							
1:30	0.080	1.040	1.030	0.060	0.190	0.080	0.120
1:150	0.017	0.580	0.540	0.022	0.070	0.020	0.027
1:750	0.009	0.280	0.260	0.010	0.020	0.010	0.014
1:3750	0.007	0.084	0.090	0.009	0.009	0.010	0.007
# Rats Tested		5	5	5	5	2	2

\* Numbers represent the average values obtained from two ELISA plates, standardized utilizing the preimmune control.

TABLE 38

Determination Of Anti-*C. botulinum* Type A Toxin Serum IgG Titers  
Following Immunization With *C. botulinum* C Fragment-Containing Fusion Proteins

Route of Immunization		Nasal		Oral	
Immunogen	PRE- IMMUNE	pMBot	pMABot	pMNABot	pMNABot
Dilution					
1:30	0.040	0.557	0.010	0.015	0.010
1:150	0.009	0.383	0.001	0.003	0.002
1:750	0.001	0.140	0.000	0.000	0.000
1:3750	0.000	0.040	0.000	0.000	0.000
# Rats Tested		1	1	3	3

The above ELISA results demonstrate that reactivity against the botulinal fusion proteins was strongest when the route of administration was nasal; only weak responses were stimulated when the botulinal fusion proteins were given orally.



Nasally delivered pMbot and pMBot admixed with pMA1870-2680 invoked the greatest serum IgG response. These results show that only the pMBot protein is necessary to induce this response, since the addition of the pMA1870-2680 protein did not enhance antibody response (Table 37). Placement of the *C. difficile* toxin A fragment between the MBP and the *C. botulinum* C fragment protein dramatically reduced anti-bot IgG titer (see results using pMABot, pMCABot and pMNABot proteins).

This study demonstrates that the pMBot protein induces a strong serum IgG response directed against *C. botulinum* type A toxin when nasally administered.

**b) In Vivo Neutralization Of *C. botulinum* Type A Neurotoxin By Anti- Recombinant *C. botulinum* C Fragment Antibodies**

The ability of the anti-*C. botulinum* type A toxin antibodies generated by nasal administration of recombinant botulinal fusion proteins in rats (Example 22) to neutralize *C. botulinum* type A toxin was tested in a mouse neutralization model. The mouse model is the art accepted method for detection of botulinal toxins in body fluids and for the evaluation of anti-botulinal antibodies [E.J. Schantz and D.A. Kautter, J. Assoc. Off. Anal. Chem. 61:96 (1990) and Investigational New Drug (BB-IND-3703) application by the Surgeon General of the Department of the Army to the Federal Food and Drug Administration]. The anti-*C. botulinum* type A toxin antibodies were prepared as follows.

Rats from the group given pMBot protein by nasal administration were boosted a second time with 250 µg pMBot protein per rat and serum was collected 7 days later. Serum from one rat from this group and from a preimmune rat was tested for anti-*C. botulinum* type A toxin neutralizing activity in the mouse neutralization model described below.

The LD<sub>50</sub> of a solution of purified *C. botulinum* type A toxin complex, obtained from Dr. Eric Johnson (University of Wisconsin Madison), was determined using the

intraperitoneal (IP) method of Schantz and Kautter [J. Assoc. Off. Anal. Chem. 61:96 (1978)] using 18-22 gram female ICR mice and was found to be 3500 LD<sub>50</sub>/ml. The determination of the LD<sub>50</sub> was performed as follows. A Type A toxin standard was prepared by dissolving purified type A toxin complex in 25 mM sodium phosphate buffer, pH 6.8 to yield a stock toxin solution of 3.15 x 10<sup>7</sup> LD<sub>50</sub>/mg. The OD<sub>278</sub> of the solution was determined and the concentration was adjusted to 10-20 µg/ml. The toxin solution was then diluted 1:100 in gel-phosphate (30 mM phosphate, pH 6.4; 0.2% gelatin). Further dilutions of the toxin solution were made as shown below in Table 39. Two mice were injected IP with 0.5 ml of each dilution shown and the mice were observed for symptoms of botulism for a period of 72 hours.

TABLE 39

Determination Of The LD<sub>50</sub> Of Purified *C. botulinum* Type A Toxin Complex

Dilution	Number Dead At 72 hr
1:320	2/2
1:640	2/2
1:1280	2/2
1:2560	0/2 (sick after 72 hr)
1:5120	0/2 (no symptoms)

From the results shown in Table 39, the toxin titer was assumed to be between 2560 LD<sub>50</sub>/ml and 5120 LD<sub>50</sub>/ml (or about 3840 LD<sub>50</sub>/ml). This value was rounded to 3500 LD<sub>50</sub>/ml for the sake of calculation.

The amount of neutralizing antibodies present in the serum of rats immunized nasally with pMBot protein was then determined. Serum from two rats boosted with pMBot protein as described above and preimmune serum from one rat was tested as follows. The toxin standard was diluted 1:100 in gel-phosphate to a final concentration of 350 LD<sub>50</sub>/ml. One milliliter of the diluted toxin standard was mixed with 25 µl of serum from each of the three rats and 0.2 ml of gel-phosphate. The mixtures were incubated at room temperature for 30 min with occasional mixing. Each of two mice were injected with IP with 0.5 ml of the mixtures. The mice were

observed for signs of botulism for 72 hr. Mice receiving serum from rats immunized with pMBot protein neutralized this challenge dose. Mice receiving preimmune rat serum died in less than 24 hr.

5 The amount of neutralizing anti-toxin antibodies present in the serum of rats immunized with pMBot protein was then quantitated. Serum antibody titrations were performed by mixing 0.1 ml of each of the antibody dilutions (see Table 40) with 0.1 ml of a 1:10 dilution of stock toxin solution ( $3.5 \times 10^4$  LD<sub>50</sub>/ml) with 1.0 ml of gel-phosphate and injecting 0.5 ml IP into 2 mice per dilution. The mice were then observed for signs of botulism for 3 days (72 hr). The results are tabulated in  
10 Table 39.

As shown in Table 40 pMBot serum neutralized *C. botulinum* type A toxin complex when used at a dilution of 1:320 or less. A mean neutralizing value of 168 IU/ml was obtained for the pMBot serum (an IU is defined as 10,000 mouse LD<sub>50</sub>). This value translates to a circulating serum titer of about 3.7 IU/mg of serum protein.  
15 This neutralizing titer is comparable to the commercially available bottled concentrated (Connaught Laboratories, Ltd.) horse anti-*C. botulinum* antiserum. A 10 ml vial of Connaught antiserum contains about 200 mg/ml of protein; each ml can neutralize 750 IU of *C. botulinum* type A toxin. After administration of one vial to a human, the circulating serum titer of the Connaught preparation would be approximately 25 IU/ml  
20 assuming an average serum volume of 3 liters). Thus, the circulating anti-*C. botulinum* titer seen in rats nasally immunized with pMBot protein (168 IU/ml) is 6.7 time higher than the necessary circulation titer of anti-*C. botulinum* antibody needed to be protective in humans.

TABLE 40

Quantitation Of Neutralizing Antibodies In pMBot Sera

Dilution	pMBot <sup>a</sup>	
	Rat 1	Rat 2
1:20	2/2	2/2
1:40	2/2	2/2
1:80	2/2	2/2
1:160	2/2	2/2
1:320	2/2 <sup>b</sup>	2/2 <sup>b</sup>
1:640	0/2	0/2
1:1280	0/2	0/2
1:2560	0/2	0/2

<sup>a</sup> Numbers represent the number of mice surviving at 72 hours which received serum taken from rats immunized with the pMBot protein.

<sup>b</sup> These mice survived but were sick after 72 hr.

These results demonstrate that antibodies capable of neutralizing *C. botulinum* type A toxin are induced when recombinant *C. botulinum* C fragment fusion protein produced in *E. coli* is used as an immunogen.

## EXAMPLE 24

Production Of Soluble *C. botulinum* C Fragment

Protein Substantially Free Of Endotoxin Contamination

Example 23 demonstrated that neutralizing antibodies are generated by immunization with the pMBot protein expressed in *E. coli*. These results showed that the pMBot fusion protein is a good vaccine candidate. However, immunogens suitable for use as vaccines should be pyrogen-free in addition to having the capability of inducing neutralizing antibodies. Expression clones and conditions that facilitate the production of *C. botulinum* C fragment protein for utilization as a vaccine were developed.

The example involved: (a) determination of pyrogen content of the pMBot protein; (b) generation of *C. botulinum* C fragment protein free of the MBP; (c) expression of *C. botulinum* C fragment protein using various expression vectors; and (d) purification of soluble *C. botulinum* C fragment protein substantially free of significant endotoxin contamination.

**a) Determination Of The Pyrogen Content Of The pMBot Protein**

In order to use a recombinant antigen as a vaccine in humans or other animals, the antigen preparation must be shown to be free of pyrogens. The most significant pyrogen present in preparations of recombinant proteins produced in gram-negative bacteria, such as *E. coli*, is endotoxin [F.C. Pearson, *Pyrogens: endotoxins, LAL testing and depyrogenation*, (1985) Marcel Dekker, New York, pp. 23-56]. To evaluate the utility of the pMBot protein as a vaccine candidate, the endotoxin content in MBP fusion proteins was determined.

The endotoxin content of recombinant protein samples was assayed utilizing the Limulus assay (LAL kit; Associates of Cape Cod ) according to the manufacturer's instructions. Samples of affinity-purified pMal-c protein and pMA1870-2680 were found to contain high levels of endotoxin [ $>50,000$  EU/mg protein; EU (endotoxin unit)]. This suggested that MBP- or toxin A repeat-containing fusions with the botulinal C fragment should also contain high levels of endotoxin. Accordingly, removal of endotoxin from affinity-purified pMal-c and pMBot protein preparations was attempted as follows.

Samples of pMal-c and pMBot protein were depyrogenated with polymyxin to determine if the endotoxin could be easily removed. The following amount of protein was treated: 29 ml at  $4.8 \text{ OD}_{280}/\text{ml}$  for pMal-c and 19 mls at  $1.44 \text{ OD}_{280}/\text{ml}$  for pMBot. The protein samples were dialyzed extensively against PBS and mixed in a 50 ml tube (Falcon) with 0.5 ml PBS-equilibrated polymyxin B (Affi-Prep Polymyxin, BioRad). The samples were allowed to mix by rotating the tubes overnight at  $4^\circ\text{C}$ .

The polymyxin was pelleted by centrifugation for 30 min in a bench top centrifuge at maximum speed (approximately 2000 x g) and the supernatant was removed. The recovered protein (in the supernatant) was quantified by OD<sub>280</sub>, and the endotoxin activity was assayed by LAL. In both cases only approximately 1/3 of the input  
5 protein was recovered and the polymyxin-treated protein retained significant endotoxin contamination (approximately 7000 EU/mg of pMBot).

The depyrogenation experiment was repeated using an independently purified pMal-c protein preparation and similar results were obtained. From these studies it was concluded that significant levels of endotoxin copurifies with these MBP fusion  
10 proteins using the amylose resin. Furthermore, this endotoxin cannot be easily removed by polymyxin treatment.

These results suggest that the presence of the MBP sequences on the fusion protein complicated the removal of endotoxin from preparations of the pMBot protein.

#### **b) Generation Of *C. botulinum* C Fragment Protein Free 15 Of The MBP**

It was demonstrated that the pMBot fusion protein could not be easily purified from contaminating endotoxin in section a) above. The ability to produce a pyrogen-free (*e.g.*, endotoxin-free) preparation of soluble botulinal C fragment protein free of the MBP tag was next investigated. The pMBot expression construct was designed to  
20 facilitate purification of the botulinal C fragment from the MBP tag by cleavage of the fusion protein by utilizing an engineered Factor Xa cleavage site present between the MBP and the botulinal C fragment. The Factor Xa cleavage was performed as follows.

Factor Xa (New England Biolabs) was added to the pMBot protein (using a  
25 0.1-1.0% Factor Xa/pMBot protein ratio) in a variety of buffer conditions [*e.g.*, PBS-NaCl (PBS containing 0.5 M NaCl), PBS-NaCl containing 0.2% Tween 20, PBS, PBS containing 0.2% Tween 20, PBS-C (PBS containing 2 mM CaCl<sub>2</sub>), PBS-C containing either 0.1 or 0.5 % Tween 20, PBS-C containing either 0.1 or 0.5% NP-40, PBS-C containing either 0.1 or 0.5% Triton X-100, PBS-C containing 0.1% sodium

deoxycholate, PBS-C containing 0.1% SDS]. The Factor Xa digestions were incubated for 12-72 hrs at room temperature.

The extent of cleavage was assessed by Western blot or Coomassie blue staining of proteins following electrophoresis on denaturing SDS-PAGE gels, as described in Example 22. Cleavage reactions (and control samples of uncleaved pMBot protein) were centrifuged for 2 min in a microfuge to remove insoluble protein prior to loading the samples on the gel. The Factor Xa treated samples were compared with uncleaved, uncentrifuged pMBot samples on the same gel. The results of this analysis is summarized below.

1) Most (about 90%) pMBot protein could be removed by centrifugation, even when uncleaved control samples were utilized. This indicated that the pMBot fusion protein was not fully soluble (*i.e.*, it exists as a suspension rather than as a solution). [This result was consistent with the observation that most affinity-purified pMBot protein precipitates after long term storage (>2 weeks) at 4°C. Additionally, the majority (*i.e.*, 75%) of induced pMBot protein remains in the pellet after sonication and clarification of the induced *E. coli*. Resuspension of these insoluble pellets in PBS followed by sonication results in partial solubilization of the insoluble pMBot protein in the pellets.]

2) The portion of pMBot protein that is fully in solution (about 10% of pMBot protein) is completely cleaved by Factor Xa, but the cleaved (released) botulinal C fragment is relatively insoluble such that only the cleaved MBP remains fully in solution.

3) None of the above reaction conditions enhanced solubility without also reducing effective cleavage. Conditions that effectively solubilized the cleaved botulinal C fragment were not identified.

4) The use of 0.1% SDS in the buffer used for Factor Xa cleavage enhanced the solubility of the pMBot protein (all of pMBot protein was soluble). However, the presence of the SDS prevented any cleavage of the fusion protein with Factor Xa.

5) Analysis of pelleted protein from the cleavage reactions indicated that both full length pMBot (*i.e.*, uncleaved) and cleaved botulinal C fragment protein precipitated during incubation.

These results demonstrate that purification of soluble botulinal C fragment protein after cleavage of the pMBot fusion protein is complicated by the insolubility of both the pMBot protein and the cleaved botulinal C fragment protein.

**c) Expression Of *C. botulinum* C Fragment Using Various Expression Vectors**

In order to determine if the solubility of the botulinal C fragment was enhanced by expressing the C fragment protein as a native protein, an N-terminal His-tagged protein or as a fusion with glutathione-S-transferase (GST), alternative expression plasmids were constructed. These expression constructs were generated utilizing the methodologies described in Example 22. Figure 27 provides a schematic representation of the vectors described below.

In Figure 27, the following abbreviations are used. pP refers to the pET23 vector. pHIS refers to the pETHisa vector. pBlue refers to the pBluescript vector. pM refers to the pMAL-c vector and pG refers to the pGEX3T vector (described in Example 11). The solid black lines represent *C. botulinum* C fragment gene sequences; the solid black ovals represent the MBP; the hatched ovals represent GST; "HHHHH" represents the poly-histidine tag. In Figure 27, when the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at a cloning junction.

**i) Construction Of pPBot**

In order to express the *C. botulinum* C fragment as a native (*i.e.*, non-fused) protein, the pPBot plasmid (shown schematically in Figure 27) was constructed as follows. The C fragment sequences present in pAlterBot (Example 22) were removed by digestion of pAlterBot with *Nco*I and *Hind*III. The *Nco*I/*Hind*III C fragment insert



was ligated to pETHisa vector (described in Example 18b) which was digested with *NcoI* and *HindIII*. This ligation creates an expression construct in which the *NcoI*-encoded methionine of the botulinal C fragment is the initiator codon and directs expression of the native botulinal C fragment. The ligation products were used to transform competent BL21(DE3)pLysS cells (Novagen). Recombinant clones were identified by restriction mapping.

## ii) Construction Of pHisBot

In order to express the *C. botulinum* C fragment containing a poly-histidine tag at the amino-terminus of the recombinant protein, the pHisBot plasmid (shown schematically in Figure 27) was constructed as follows. The *NcoI/HindIII* botulinal C fragment insert from pAlterbot was ligated into the pETHisa vector which was digested with *NheI* and *HindIII*. The *NcoI* (on the C fragment insert) and *NheI* (on the pETHisa vector) sites were filled in using the Klenow fragment prior to ligation; these sites were then blunt end ligated (the *NdeI* site was regenerated at the clone junction as predicted). The ligation products were used to transform competent BL21(DE3)pLysS cells and recombinant clones were identified by restriction mapping.

The resulting pHisBot clone expresses the botulinal C fragment protein with a histidine-tagged N-terminal extension having the following sequence: MetGlyHisHisHisHisHisHisHisHisHisSerSerGlyHisIleGluGlyArgHisMetAla, (SEQ ID NO:24); the amino acids encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type. The nucleotide sequence present in the pETHisa vector which encodes the pHisBot fusion protein is listed in SEQ ID NO:25. The amino acid sequence of the pHisBot protein is listed in SEQ ID NO:26.

### iii) Construction Of pGBot

The botulinal C fragment protein was expressed as a fusion with the glutathione-S-transferase protein by constructing the pGBot plasmid (shown schematically in Figure 27). This expression construct was created by cloning the  
5 *NotI/SaII* C fragment insert present in pBlueBot (Example 22) into the pGEX3T vector which was digested with *SmaI* and *XhoI*. The *NotI* site (present on the botulinal fragment) was made blunt prior to ligation using the Klenow fragment. The ligation products were used to transform competent BL21 cells.

Each of the above expression constructs were tested by restriction digestion to  
10 confirm the integrity of the constructs.

Large scale (1 liter) cultures of pPBot [BL21(DE3)pLysS host], pHisBot [BL21(DE3)pLysS host] and pGBot (BL21 host) were grown in 2X YT medium and induced (using IPTG to 0.8-1.0 mM) for 3 hrs as described in Example 22. Total, soluble and insoluble protein preparations were prepared from 1 ml aliquots of each  
15 large scale culture [Williams *et al.* (1994), *supra*] and analyzed by SDS-PAGE. No obvious induced band was detectable in the pPBot or pHisBot samples by Coomassie staining, while a prominent insoluble band of the anticipated MW was detected in the pGBot sample. Soluble lysates of the pGBot large scale (resuspended in PBS) or pHisBot large scale [resuspended in Novagen 1X binding buffer (5 mM imidazole, 0.5  
20 M NaCl, 20 mM Tris-HCl, pH 7.9)] cultures were prepared and used to affinity purify soluble affinity-tagged protein as follows.

The pGBot lysate was affinity purified on a glutathione-agarose resin (Pharmacia) exactly as described in Smith and Corcoran [Current Protocols in Molecular Biology, Supplement 28 (1994), pp. 16.7.1-16.7.7]. The pHisBot protein  
25 was purified on the His-Bind resin (Novagen) utilizing the His-bind buffer kit (Novagen) exactly as described by manufacturer.

Samples from the purification of both the pGBot and pHisBot proteins (including uninduced, induced, total, soluble, and affinity-purified eluted protein) were resolved on SDS-PAGE gels. Following electrophoresis, proteins were analyzed by

Coomassie staining or by Western blot detection utilizing a chicken anti-*C. botulinum* Type A toxoid antibody (as described in Example 22).

These studies showed that the pGBot protein was almost entirely insoluble under the utilized conditions, while the pHisBot protein was soluble. Affinity purification of the pHisBot protein on this first attempt was inefficient, both in terms of yield (most of the immunoreactive botulin protein did not bind to the His-bind resin) and purity (the botulin protein was estimated to comprise approximately 20% of the total eluted protein).

**d) Purification Of Soluble *C. botulinum* C Fragment  
Protein Substantially Free Of Endotoxin  
Contamination**

The above studies showed that the pHisBot protein was expressed in *E. coli* as a soluble protein. However, the affinity purification of this protein on the His-bind resin was very inefficient. In order to improve the affinity purification of the soluble pHisBot protein (in terms of both yield and purity), an alternative poly-histidine binding affinity resin (Ni-NTA resin; Qiagen) was utilized. The Ni-NTA resin was reported to have a superior binding affinity ( $K_d = 1 \times 10^{-13}$  at pH 8.0; Qiagen user manual) relative to the His-bind resin.

A soluble lysate (in Novagen 1X binding buffer) from an induced 1 liter 2X YT culture was prepared as described above. Briefly, the culture of pHisBot [BI21(DE3)pLysS host] was grown at 37°C to an  $OD_{600}$  of 0.7 in 1 liter of 2X YT medium containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 0.2% glucose. Protein expression was induced by the addition of IPTG to 1 mM. Three hours after the addition of the IPTG, the cells were cooled for 15 min in a ice water bath and then centrifuged 10 min at 5000 rpm in a JA10 rotor (Beckman) at 4°C. The pellets were resuspended in a total volume of 40 mls Novagen 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), transferred to two 35 ml Oakridge tubes and frozen at -70°C for at least 1 hr. The tubes were thawed and the cells were lysed by sonication (4 X 20 second bursts using a Branson Sonifier 450

with a power setting of 6-7) on ice. The suspension was clarified by centrifugation for 20 min at 9,000 rpm (10,000 x g) in a JA-17 rotor (Beckman).

5 The soluble lysate was brought to 0.1% NP40 and then was batch absorbed to 7 ml of a 1:1 slurry of Ni-NTA resin:binding buffer by stirring for 1 hr at 4°C. The slurry was poured into a column having an internal diameter of 1 or 2.5 cm (BioRad). The column was then washed sequentially with 15 mls of Novagen 1X binding buffer containing 0.1% NP40, 15 ml of Novagen 1X binding buffer, 15 ml wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and 15 ml NaHPO<sub>4</sub> wash  
10 buffer (50 mM NaHPO<sub>4</sub>, pH 7.0, 0.3 M NaCl, 10 % glycerol). The bound protein was eluted by protonation of the resin using elution buffer (50 mM NaHPO<sub>4</sub>, pH 4.0, 0.3 M NaCl, 10 % glycerol). The eluted protein was stored at 4°C.

Samples of total, soluble and eluted protein were resolved by SDS-PAGE. Protein samples were prepared for electrophoresis as described in Example 22b. Duplicate gels were stained with Coomassie blue to visualize the resolved proteins and  
15 *C. botulinum* type A toxin-reactive protein was detected by Western blot analysis as described in Example 22b. A representative Coomassie stained gel is shown in Figure 28. In Figure 28, the following samples were loaded on the 12.5% acrylamide gel. Lanes 1-4 contain respectively total protein, soluble protein, soluble protein present in the flow-through of the Ni-NTA column and affinity-purified pHisBot  
20 protein (*i.e.*, protein released from the Ni-NTA resin by protonation). Lane 5 contains high molecular weight protein markers (BioRad).

The purification of pHisBot protein resulted in a yield of 7 mg of affinity purified protein from a 1 liter starting culture of BL21(DE3)pLysS cells harboring the pHisBot plasmid. The yield of purified pHisBot protein represented approximately  
25 0.4% of the total soluble protein in the induced culture. Analysis of the purified pHisBot protein by SDS-PAGE revealed that at least 90-95% of the protein was present as a single band (Figure 28) of the predicted MW (50 kD). This 50 kD protein band was immunoreactive with anti-*C. botulinum* type A toxin antibodies. The extinction coefficient of the protein preparation was determined to be 1.4 (using the  
30 Pierce BCA assay) or 1.45 (using the Lowry assay) OD<sub>280</sub> per 1 mg/ml solution.

Samples of pH neutralized eluted pHisBot protein were resolved on a KB 803 HPLC column (Shodex). Although His-tagged proteins are retained by this sizing column (perhaps due to the inherent metal binding ability of the proteins), the relative mobility of the pHisBot protein was consistent with that expected for a non-aggregated protein in solution. Most of the induced pHisBot protein was determined to be soluble under the growth and solubilization conditions utilized above (*i.e.*, greater than 90% of the pHisBot protein was found to be soluble as judged by comparison of the levels of pHisBot protein seen in total and soluble protein samples prepared from BL21(DE3)pLysS cells containing the pHisBot plasmid). SDS-PAGE analysis of samples obtained after centrifugation, extended storage at -20°C, and at least 2 cycles of freezing and thawing detected no protein loss (due to precipitation), indicating that the pHisBot protein is soluble in the elution buffer (*i.e.*, 50 mM NaHPO<sub>4</sub>, pH 4.0, 0.3 M NaCl, 10 % glycerol).

Determination of endotoxin contamination in the affinity purified pHisBot preparation (after pH neutralization) using the LAL assay (Associates of Cape Cod) detected no significant endotoxin contamination. The assay was performed using the endpoint chromogenic method (without diazo-coupling) according to the manufacturer's instructions. This method can detect concentrations of endotoxin greater than or equal to 0.03 EU/ml (EU refers to endotoxin units). The LAL assay was run using 0.5 ml of a solution comprising 0.5 mg pHisBot protein in 50 mM NaHPO<sub>4</sub>, pH 7.0, 0.3 M NaCl, 10 % glycerol; 30-60 EU were detected in the 0.5 ml sample. Therefore, the affinity purified pHisBot preparation contains 60-120 EU/mg of protein. FDA Guidelines for the administration of parenteral drugs require that a composition to be administered to a human contain less than 5 EU/kg body weight (The average human body weight is 70 kg; therefore up to 349 EU units can be delivered in a parental dose.). Because very small amount of protein are administered in a vaccine preparation (generally in the range of 10-500 µg of protein), administration of affinity purified pHisBot containing 60-120 EU/mg protein would result in delivery of only a small percentage of the permissible endotoxin load. For example, administration of 10-500 µg of purified pHisBot to a 70 kg human, where

the protein preparation contains 60 EU/mg protein, results in the introduction of only 0.6 to 30 EU [*i.e.*, 0.2 to 8.6% of the maximum allowable endotoxin burden per parenteral dose (less than 5 EU/kg body weight)].

5 The above results demonstrate that endotoxin (LPS) does not copurify with the pHisBot protein using the above purification scheme. Preparations of recombinantly produced pHisBot protein containing lower levels of endotoxin (less than or equal to 2 EU/ mg recombinant protein) may be produced by washing the Ni-NTA column with wash buffer until the OD<sub>280</sub> returns to baseline levels (*i.e.*, until no more UV-absorbing material comes off of the column).

10 The above results illustrate a method for the production and purification of soluble, botulinal C fragment protein substantially free of endotoxin.

## EXAMPLE 25

### Optimization Of The Expression And Purification Of pHisBot Protein

15 The results shown in Example 24d demonstrated that the pHisBot protein is an excellent candidate for use as a vaccine as it could be produced as a soluble protein in *E. coli* and could be purified free of pyrogen activity. In order to optimize the expression and purification of the pHisBot protein, a variety of growth and purification conditions were tested.

#### a) Growth Parameters

20 i) Host Strains

The influence of the host strain utilized upon the production of soluble pHisBot protein was investigated. A large scale purification of pHisBot was performed [as described in Example 24d above] using the BL21(DE3) host (Novagen) rather than the BL21(DE3)pLysS host. The deletion of the pLysS plasmid in the BL21(DE3) host  
25 yielded higher levels of expression due to de-repression of the plasmid's T7-lac promoter. However, the yield of affinity-purified soluble recombinant protein was very low (approximately 600 µg/ liter culture) when purified under conditions identical

to those described in Example 24d above. This result was due to the fact that expression in the BL21(DE3) host yielded very high level expression of the pHisBot protein as insoluble inclusion bodies as shown by SDS-PAGE analysis of protein prepared from induced BL21(DE3) cultures (Figure 29, lanes 1-7, described below).

5 These results demonstrate that the pHisBot protein is not inherently toxic to *E. coli* cells and can be expressed to high levels using the appropriate promoter/host combination.

Figure 29 shows a Coomassie blue stained SDS-PAGE gel (12.5% acrylamide) onto which extracts prepared from BL21(DE3) cells containing the pHisBot plasmid were loaded. Each lane was loaded with 2.5  $\mu$ l protein sample mixed with 2.5  $\mu$ l of 10 2X SDS sample buffer. The samples were handled as described in Example 22b. The following samples were applied to the gel. Lanes 1-7 contain protein isolated from the BL21(DE3) host. Lanes 8-14 contain proteins isolated from the BL21(DE3)pLysS host. Total protein was loaded in lanes 1, 2, 4, 6, 8, 10 and 12. Soluble protein was 15 loaded in Lanes 3, 5, 7, 9, 11 and 13. Lane 1 contains protein from uninduced host cells. Lanes 2-13 contain protein from host cells induced for 3 hours. IPTG was added to a final concentration of 0.1 mM (Lanes 6-7), 0.3 mM (Lanes 4-5) or 1.0 mM (Lanes 2, 3, 8-13). The cultures were grown in LB broth (Lanes 8-9), 2X YT broth (Lanes 10-11) or terrific broth (Lanes 1-7, 12-13). The pHisBot protein seen in Lanes 20 3, 5 and 7 is insoluble protein which spilled over from Lanes 2, 4 and 6, respectively. High molecular weight protein markers (BioRad) were loaded in Lane 14.

A variety of expression conditions were tested to determine if the BL21(DE3) host could be utilized to express soluble pHisBot protein at suitably high levels (*i.e.*, about 10 mg/ml). The conditions altered were temperature (growth at 37 or 30°C), 25 culture medium (2X YT, LB or Terrific broth) and inducer levels (0.1, 0.3 or 1.0 mM IPTG). All combinations of these variables were tested and the induction levels and solubility was then assessed by SDS-PAGE analysis of total and soluble extracts [prepared from 1 ml samples as described in Williams *et al.*, (1994), *supra*].

All cultures were grown in 15 ml tubes (Falcon #2057). All culture medium 30 was prewarmed overnight at the appropriate temperature and were supplemented with

100 µg/ml ampicillin and 0.2% glucose. Terrific broth contains 12 g/l bacto-tryptone, 24 g/l bacto-yeast extract and 100 ml/l of a solution comprising 0.17 M  $\text{KH}_2\text{PO}_4$ , 0.72 M  $\text{K}_2\text{HPO}_4$ . Cultures were grown in a incubator on a rotating wheel (to ensure aeration) to an  $\text{OD}_{600}$  of approximately 0.4, and induced by the addition of IPTG. In all cases, high level expression of insoluble pHisBot protein was observed, regardless of temperature, medium or inducer concentration.

The effect of varying the concentration of IPTG upon 2X YT cultures grown at 23°C was then investigated. IPTG was added to a final concentration of either 1 mM, 0.1 mM, 0.05 mM or 0.01 mM. At this temperature, similar levels of pHis Bot protein was induced in the presence of either 1 or 0.1 mM IPTG; these levels of expression was lower than that observed at higher temperatures. Induced protein levels were reduced at 0.05 mM IPTG and absent at 0.01 mM IPTG (relative to 1.0 and 0.1 mM IPTG inductions at 23°C). However, no conditions were observed in which the induced pHisBot protein was soluble in this host. Thus, although expression levels are superior in the BL21(DE3) host (as compared to the BL21(DE3)pLysS host), conditions that facilitate the production of soluble protein in this host could not be identified.

These results demonstrate that production of soluble pHisBot protein was achieved using the BL21(DE3)pLysS host in conjunction with the T7-lac promoter.

#### **ii) Effect Of Varying Temperature, Medium And IPTG Concentration And Length Of Induction**

The effect growing the host cells in various mediums upon the expression of recombinant botulinal protein from the pHisBot expression construct [in the BL21(DE3)pLysS host] was investigated. BL21(DE3)pLysS cells containing the pHisBot plasmid were grown in either LB, 2X YT or Terrific broth at 37°C. The cells were induced using 1 mM IPTG for a 3 hr induction period. Expression of pHisBot protein was found to be the highest when the cells were grown in 2X YT broth (see Figure 29, lanes 8-13).



The cells were then grown at 30°C in 2X YT broth and the concentration of IPTG was varied from 1.0, 0.3 or 0.1 mM and the length of induction was either 3 or 5 hours. Expression of pHisBot protein was similar at all 3 inducer concentrations utilized and the levels of induced protein were higher after a 5 hr induction as compared to a 3 hr induction.

Using the conditions found to be optimal for the expression of pHisBot protein, a large scale culture was grown in order to provide sufficient material for a large scale purification of the pHisBot protein. Three 1 liter cultures were grown in 2X YT medium containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 0.2% glucose. The cultures were grown at 30°C and were induced with 1.0 mM IPTG for a 5 hr period. The cultures were harvested and a soluble lysate were prepared as described in Example 18. A large scale purification was performed as described in Example 24d with the exception that except the soluble lysate was batch absorbed for 3 hours rather than for 1 hour. The final yield was 13 mg pHisBot protein/liter culture. The pHisBot protein represented 0.75% of the total soluble protein.

The above results demonstrate growth conditions under which soluble pHisBot protein is produced (*i.e.*, use of the BL21(DE3)pLysS host, 2X YT medium, 30°C, 1.0 mM IPTG for 5 hours).

#### **b) Optimization Of Purification Parameters**

For optimization of purification conditions, large scale cultures (3 X 1 liter) were grown at 30°C and induced with 1 mM IPTG for 5 hours as described above. The cultures were pooled, distributed to centrifuge bottles, cooled and pelleted as described in Example 24d. The cell pellets were frozen at -70°C until used. Each cell pellet represented 1/3 of a liter starting culture and individual bottles were utilized for each optimization experiment described below. This standardized the input bacteria used for each experiment, such that the yields of affinity purified pHisBot protein could be compared between different optimization experiments.

**i) Binding Specificity (pH Protonation)**

5 A lysate of pHisBot culture was prepared in PBS (pH 8.0) and applied to a 3 ml Ni-NTA column equilibrated in PBS (pH 8.0) using a flow rate of 0.2 ml/min (3-4 column volumes/hr) using an Econo chromatography system (BioRad). The column was washed with PBS (pH 8.0) until the absorbance ( $OD_{280}$ ) of the elute was at baseline levels. The flow rate was then increased to 2 ml/min and the column was equilibrated in PBS (pH 7.0). A pH gradient (pH 7.0 to 4.0 in PBS) was applied in order to elute the bound pHisBot protein from the column. Fractions were collected and aliquots were resolved on SDS-PAGE gels. The PAGE gels were subjected to Western blotting and the pHisBot protein was detected using a chicken anti-*C. botulinum* Type A toxoid antibody as described in Example 22.

10 From the Western blot analysis it was determined that the pHisBot protein begins to elute from the Ni-NTA column at pH 6.0. This is consistent with the predicted elution of a His-tagged protein monomer at pH 5.9.

15 These results demonstrate that the pH at which the pHisBot protein is protonated (released) from Ni-NTA resin in PBS buffer is pH 6.0.

**ii) Binding Specificity (Imidazole Competition)**

20 In order to define purification conditions under which the native *E. coli* proteins could be removed from the Ni-NTA column while leaving the pHisBot protein bound to the column, the following experiment was performed. A lysate of pHisBot culture was prepared in 50 mM  $\text{NaHPO}_4$ , 0.5 M NaCl, 8 mM imidazole (pH 7.0). This lysate was applied to a 3 ml Ni-NTA column equilibrated in 50 mM  $\text{NaHPO}_4$ , 0.5 M NaCl (pH 7.0) using an Econo chromatography system (BioRad). A flow rate of 0.2 ml/min (3-4 column volumes/hr) was utilized. The column was washed with 50 mM  $\text{NaHPO}_4$ , 0.5 M NaCl (pH 7.0) until the absorbance of the elute returned to baseline. The flow rate was then increased to 2 ml/min.

25 The column was eluted using an imidazole step gradient [in 50 mM  $\text{NaHPO}_4$ , 0.5 M NaCl (pH 7.0)]. Elution steps were 20 mM, 40 mM, 60 mM, 80 mM, 100 mM,

200 mM, 1.0 M imidazole, followed by a wash using 0.1 mM EDTA (to strip the nickel from the column and remove any remaining protein). In each step, the wash was continued until the OD<sub>280</sub> returned to baseline. Fractions were resolved on SDS-PAGE gels, Western blotted, and pHisBot protein detected using a chicken anti-*C. botulinum* Type A toxoid antibody as described in Example 22. Duplicate gels were stained with Coomassie blue to detect eluted protein in each fraction.

The results of the PAGE analysis showed that most of the non-specifically binding bacterial protein was removed by the 20 mM imidazole wash, with the remaining bacterial proteins being removed in the 40 and 60 mM imidazole washes. The pHisBot protein began to elute at 100 mM imidazole and was quantitatively eluted in 200 mM imidazole.

These results precisely defined the window of imidazole wash stringency that optimally removes *E. coli* proteins from the column while specifically retaining the pHisBot protein in this buffer. These results provided conditions under which the pHisBot protein can be purified free of contaminating host proteins.

### iii) Purification Buffers And Optimized Purification Protocols

A variety of purification parameters were tested during the development of an optimized protocol for batch purification of soluble pHisBot protein. The results of these analyses are summarized below.

Batch purifications were performed (as described in Example 24d) using several buffers to determine if alternative buffers could be utilized for binding of the pHisBot protein to the Ni-NTA column. It was determined that quantitative binding of pHisBot protein to the Ni-NTA resin was achieved in either Tris-HCl (pH 7.9) or NaHPO<sub>4</sub> (pH 8.0) buffers. Binding of the pHisBot protein in NaHPO<sub>4</sub> buffer was not inhibited using 5 mM, 8 mM or 60 mM imidazole. Quantitative elution of bound pHisBot protein was obtained in buffers containing 50 mM NaHPO<sub>4</sub>, 0.3 M NaCl (pH 3.5-4.0), with or without 10% glycerol. However, quantitation of soluble affinity purified pHisBot protein before and after a freeze thaw (following several weeks storage of the affinity

purified elute at -20°C) revealed that 94% of the protein was recovered using the glycerol-containing buffer, but only 68% of the protein was recovered when the buffer lacking glycerol was employed. This demonstrates that glycerol enhanced the solubility of the pHisBot protein in this low pH buffer when the eluted protein was stored at freezing temperatures (*e.g.*, -20°C). Neutralization of pH by addition of NaH<sub>2</sub>PO<sub>4</sub> buffer did not result in obvious protein precipitation.

It was determined that quantitative binding of pHisBot protein using the batch format occurred after 3 hrs (Figure 30), but not after 1 hr of binding at 4°C (the resin was stirred during binding). Figure 30 depicts a Coomassie blue stained SDS-PAGE gel (7.5% acrylamide) containing samples of proteins isolated during the purification of pHisBot protein from lysate prepared from the BL21(DE3)pLysS host. Each lane was loaded with 5 µl of protein sample mixed with 5 µl of 2X sample buffer and processed as described in Example 22b. Lane 1 contains high molecular weight protein markers (BioRad). Lanes 2 and 3 contain protein eluted from the Ni-NTA resin. Lane 4 contains soluble protein after a 3 hr batch incubation with the Ni-NTA resin. Lanes 5 and 6 contain soluble and total protein, respectively. Figure 30 demonstrates that the pHisBot protein is completely soluble [compare Lanes 5 and 6 which show that a similar amount of the 50 kD pHisBot protein is seen in both; if a substantial amount (greater than 20%) of the pHisBot protein were partially insoluble in the host cell, more pHisBot protein would be seen in lane 6 (total protein) as compared to lane 5 (soluble protein)]. Figure 30 also demonstrates that the pHisBot protein is completely removed from the lysate after batch absorption with the Ni-NTA resin for 3 hours (compare Lanes 4 and 5).

The reported high affinity interaction of the Ni-NTA resin with His-tagged proteins ( $K_d = 1 \times 10^{-13}$  at pH 8.0) suggested that it should be possible to manipulate the resin-protein complexes without significant release of the bound protein. Indeed, it was determined that after the recombinant protein was bound to the Ni-NTA resin, the resin-pHisBot protein complex was highly stable and remained bound following repeated rounds of centrifugation of the resin for 2 min at 1600 x g. When this centrifugation step was performed in a 50 ml tube (Falcon), a tight resin pellet formed.

This allowed the removal of spent soluble lysate by pouring off the supernatant followed by resuspension of the pellet in wash buffer. Further washes can be performed by centrifugation. The ability to perform additional washes permits the development of protocols for batch absorption of large volumes of lysate with removal of the lysate being performed simply by centrifugation following binding of the recombinant protein to the resin.

A simplified, integrated purification protocol was developed as follows. A soluble lysate was made by resuspending the induced cell pellet in binding buffer [50 mM NaHPO<sub>4</sub>, 0.5 M NaCl, 60 mM imidazole (pH 8.0)], sonicating 4 x 20 sec and centrifuging for 20 min at 10,000 x g. NP-40 was added to 0.1% and Ni-NTA resin (equilibrated in binding buffer) was added. Eight milliliters of a 1:1 slurry (resin:binding buffer) was used per liter of starting culture. The mixture was stirred for 3 hrs at 4°C. The slurry was poured into a column having a 1 cm internal diameter (BioRad), washed with binding buffer containing 0.1% NP40, then binding buffer until baseline was established (these steps may alternatively be performed by centrifugation of the resin, resuspension in binding buffer containing NP40 followed by centrifugation and resuspension in binding buffer). Imidazole was removed by washing the resin with 50 mM NaHPO<sub>4</sub>, 0.3M NaCl (pH 7.0). Protein bound to the resin was eluted using the same buffer (50 mM NaHPO<sub>4</sub>, 0.3M NaCl) having a reduced pH (pH 3.5-4.0).

A pilot purification was performed following this protocol and yielded 18 mg/liter affinity-purified pHisBot. The pHisBot protein was greater than 90% pure as estimated by Coomassie staining of an SDS-PAGE gel. This represents the highest observed yield of soluble affinity-purified pHisBot protein and this protocol eliminates the need for separate imidazole-containing binding and wash buffers. In addition to providing a simplified and efficient protocol for the affinity purification of recombinant pHisBot protein, the above results provide a variety of purification conditions under which pHisBot protein can be isolated.

## EXAMPLE 26

### The pHisBot Protein Is An Effective Immunogen

In Example 23 it was demonstrated that neutralizing antibodies are generated in mouse serum after nasal immunization with the pMBot protein. However, the pMBot protein was found to copurify with significant amounts of endotoxin which could not be easily removed. The pHisBot protein, in contrast, could be isolated free of significant endotoxin contamination making pHisBot a superior candidate for vaccine production. To further assess the suitability of pHisBot as a vaccine, the immunogenicity of the pHisBot protein was determined and a comparison of the relative immunogenicity of pMBot and pHisBot proteins in mice was performed as follows.

Two groups of eight BALBc mice were immunized with either pMBot protein or pHisBot protein using Gerbu GMDP adjuvant (CC Biotech). pMBot protein (in PBS containing 10 mM maltose) or pHisBot protein (in 50 mMNaHPO<sub>4</sub>, 0.3 M NaCl, 10% glycerol, pH 4.0) was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received an IP injection of 100 µl antigen/adjuvant mix (50 µg antigen plus 1 µg adjuvant) on day 0. Mice were boosted as described above with the exception that the route of administration was IM on day 14 and 28. The mice were bled on day 77 and anti-*C. botulinum* Type A toxoid titers were determined using serum collected from individual mice in each group (as described in Example 23). The results are shown in Table 41.

TABLE 41

Anti-*C. botulinum* Type A Toxoid Serum IgG Titers In Individual Mice Immunized With pMBot or pHisBot Protein

Mouse #	Preimmune <sup>1</sup>				pMBot <sup>2</sup>				pHisBot <sup>2</sup>			
	Sample Dilution				Sample Dilution				Sample Dilution			
	1:50	1:250	1:1250	1:6250	1:50	1:250	1:1250	1:6250	1:50	1:250	1:1250	1:620
1					0.678	0.190	0.055	0.007	1.574	0.799	0.320	0.093
2					1.161	0.931	0.254	0.075	1.513	0.829	0.409	0.134
3					1.364	0.458	0.195	0.041	1.596	1.028	0.453	0.122
4					1.622	1.189	0.334	0.067	1.552	0.840	0.348	0.090
5					1.612	1.030	0.289	0.067	1.629	1.580	0.895	0.233
6					0.913	0.242	0.069	0.013	1.485	0.952	0.477	0.145
7					0.910	0.235	0.058	0.014	1.524	0.725	0.269	0.069
8					0.747	0.234	0.058	0.014	1.274	0.427	0.116	0.029
Mean Titer	0.048	0.021	0.011	0.002	1.133	0.564	0.164	0.037	1.518	0.896	0.411	0.114

<sup>1</sup> The preimmune sample represents the average from 2 sets of duplicate wells containing serum from a individual mouse immunized with recombinant *Staphylococcus* enterotoxin B (SEB) antigen. This antigen is immunologically unrelated to *C. botulinum* toxin and provides a control serum.

<sup>2</sup> Average of duplicate wells.

The results shown above in Table 41 demonstrate that both the pMBot and pHisBot proteins are immunogenic in mice as 100% of the mice (8/8) in each group seroconverted from non-immune to immune status. The results also show that the average titer of anti-*C. botulinum* Type A toxoid IgG is 2-3 fold higher after immunization with the pHisBot protein relative to immunization with the pMBot protein. This suggests that the pHisBot protein may be a superior immunogen to the pMBot protein.

## EXAMPLE 27

### Immunization With The Recombinant pHisBot Protein Generates Neutralizing Antibodies

5 The results shown in Example 26 demonstrated that both the pHisBot and pMBot proteins were capable of inducing high titers of anti-*C. botulinum* type A toxoid-reactive antibodies in immunized hosts. The ability of the immune sera from mice immunized with either the pHisBot or pMBot proteins to neutralize *C. botulinum* type A toxoid *in vivo* was determined using the mouse neutralization assay described in Example 23b.

10 The two groups of eight BALBc mice immunized with either pMBot protein or pHisBot protein in Example 26 were boosted again one week after the bleeding on day 77. The boost was performed by mixing pMBot protein (in PBS containing 10 mM maltose) or pHisBot protein (in 50 mM NaHPO<sub>4</sub>, 0.3 M NaCl, 10% glycerol, pH 4.0) with Gerbu adjuvant as described in Example 26. Each mouse received an IP injection  
15 of 100 µl antigen/adjuvant mix (50 µg antigen plus 1 µg adjuvant). The mice were bled 6 days after this boost and the serum from mice within a group was pooled. Serum from preimmune mice was also collected (this serum is the same serum described in the footnote to Table 41).

20 The presence of neutralizing antibodies in the pooled or preimmune serum was detected by challenging mice with 5 LD<sub>50</sub> units of type A toxin mixed with 100 µl of pooled serum. The challenge was performed by mixing (per mouse to be injected) 100 µl of serum from each pool with 100 µl of purified type A toxin standard (50 LD<sub>50</sub> /ml prepared as described in Example 23b) and 500 µl of gel-phosphate. The mixtures  
25 were incubated for 30 min at room temperature with occasional mixing. Each of four mice were injected IP with the mixtures (0.7 ml/mouse). The mice were observed for signs of botulism for 72 hours. Mice receiving toxin mixed with serum from mice immunized with either the pHisBot or pMBot proteins showed no signs of botulism intoxication. In contrast, mice receiving preimmune serum died in less than 24 hours.



These results demonstrate that antibodies capable of neutralizing *C. botulinum* type A toxin are induced when either of the recombinant *C. botulinum* C fragment proteins pHisBot or pMBot are used as immunogens.

#### EXAMPLE 28

5                    Cloning And Expression Of The C Fragment of *C. botulinum*  
                    Serotype A Toxin In *E. coli* Utilizing A Native Gene Fragment

10                   In Example 22 above, a synthetic gene was used to express the C fragment of  
*C. botulinum* serotype A toxin in *E. coli*. The synthetic gene replaced non-preferred  
(*i.e.*, rare) codons present in the C fragment gene with codons which are preferred by  
*E. coli*. The synthetic gene was generated because it has been reported that genes  
which have a high A/T content (such as most clostridial genes) creates expression  
difficulties in *E. coli* and yeast. Furthermore, LaPenotiere *et al.* suggested that  
problems encountered with the stability (non-fusion constructs) and solubility (MBP  
fusion constructs) of the C fragment of *C. botulinum* serotype A toxin when expressed  
15 in *E. coli* was most likely due to the extreme A/T richness of the native *C. botulinum*  
serotype A toxin gene sequences (LaPenotiere, *et al.*, *supra*).

20                   In this example, it was demonstrated that successful expression of the C  
fragment of *C. botulinum* type A toxin gene in *E. coli* does not require the elimination  
of rare codons (*i.e.*, there is no need to use a synthetic gene). This example involved  
a) the cloning of the native C fragment of the *C. botulinum* serotype A toxin gene and  
construction of an expression vector and b) a comparison of the expression and  
purification yields of *C. botulinum* serotype A C fragments derived from native and  
synthetic expression vectors.

a) **Cloning Of The Native C Fragment Of The  
*C. botulinum* Serotype A Toxin Gene And  
Construction Of An Expression Vector**

The serotype A toxin gene was cloned from *C. botulinum* genomic DNA using  
5 PCR amplification. The following primer pair was employed: 5'-CGCCATGGCTAG  
ATTATTATCTACATTTAC-3' (5' primer, *Nco*I site underlined; SEQ ID NO:29) and  
5'-GCAAGCTTCTTGACAGACTCATGTAG-3' (3' primer, *Hind*III site underlined;  
SEQ ID NO:30). *C. botulinum* type A strain was obtained from the American Type  
Culture Collection (ATCC#19397) and grown under anaerobic conditions in Terrific  
10 broth medium. High molecular-weight *C. botulinum* DNA was isolated as described in  
Example 11. The integrity and yield of genomic DNA was assessed by comparison  
with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

The gene fragment was cloned by PCR utilizing a proofreading thermostable  
DNA polymerase (native *Pfu* polymerase). PCR amplification was performed using  
15 the above primer pair in a 50 $\mu$ l reaction containing 10mM Tris-HCl (pH 8.3), 50mM  
KCl, 1.5mM MgCl<sub>2</sub>, 200 $\mu$ M each dNTP, 0.2 $\mu$ M each primer, and 50ng *C. botulinum*  
genomic DNA. Reactions were overlaid with 100 $\mu$ l mineral oil, heated to 94°C 4 min,  
0.5 $\mu$ l native *Pfu* polymerase (Stratagene) was added, and thirty cycles comprising 94°C  
for 1 min, 50°C for 2 min, 72°C for 2 min were carried out followed by 10 min at  
20 72°C. An aliquot (10 $\mu$ l) of the reaction mixture was resolved on an agarose gel and  
the amplified native C fragment gene was gel purified using the Prep-A-Gene kit  
(BioRad) and ligated to pCRScript vector DNA (Stratagene). Recombinant clones  
were isolated and confirmed by restriction digestion, using standard recombinant  
molecular biology techniques [Sambrook *et al.* (1989), *supra*]. In addition, the  
25 sequence of approximately 300 bases located at the 5' end of the C fragment coding  
region were obtained using standard DNA sequencing methods. The sequence  
obtained was identical to that of the published sequence.

An expression vector containing the native *C. botulinum* serotype A C fragment  
gene was created by ligation of the *Nco*I-*Hind*III fragment containing the C fragment

gene from the pCRScript clone to *NheI-HindIII* restricted pETHisa vector (Example 18b). The *NcoI* and *NheI* sites were filled in using the Klenow enzyme prior to ligation; these sites were thus blunt-end ligated together. The resulting construct was termed pHisBotA (native). pHisBotA (native) expresses the *C. botulinum* serotype A C fragment with a his-tagged N terminal extension which has the following sequence: MetGlyHisHisHisHisHisHisHisHisHisSerSerGlyHisIleGluGlyArgHisMetAla (SEQ ID NO:24), where the underlining represents amino acids encoded by the *C. botulinum* C fragment gene (this N terminal extension contains the recognition site for FactorXa protease, shown in italics, which can be employed to removed the polyhistidine tract from the N-terminus of the fusion protein). The pHisBot (native) construct expresses the identical protein as the pHisBot construct (Ex. 24c; herein after the pHisBotA) which contains the synthetic gene.

The predicted DNA sequence encoding the native *C. botulinum* serotype A C fragment gene contained within pHisBotA (native) is listed in SEQ ID NO:31 [the start of translation (ATG) is located at nucleotides 108-110 and the stop of translation (TAA) is located at nucleotides 1494-1496 in SEQ ID NO:31] and the corresponding amino acid sequence is listed in SEQ ID NO:26 (*i.e.*, the same amino acid sequence as that produced by pHisBotA containing synthetic gene sequences).

**b) Comparison Of The Expression And Purification  
Yields Of *C. botulinum* Serotype A C Fragments  
Derived From Native And Synthetic Expression  
Vectors**

Recombinant plasmids containing either the native or the synthetic *C. botulinum* serotype A C fragment genes were transformed into *E. coli* strain B121(DE3) pLysS and protein expression was induced in 1 liter shaker flask cultures. Total protein extracts were isolated, resolved on SDS-PAGE gels and *C. botulinum* C fragment protein was identified by Western analysis utilizing a chicken anti-*C. botulinum* serotype A toxoid antiserum as described in Example 22.

Briefly, 1 liter (2XYT + 100  $\mu$ g/ml ampicillin and 34  $\mu$ g/ml chloramphenicol) cultures of bacteria harboring either the pHisBotA (synthetic) or pHisBotA (native) plasmids in the B121(DE3) pLysS strain were induced to express recombinant protein by addition of IPTG to 1mM. Cultures were grown at 30-32°C, IPTG was added  
5 when the cell density reached an OD<sub>600</sub> 0.5-1.0 and the induced protein was allowed to accumulate for 3-4 hrs after induction.

The cells were cooled for 15 min in a ice water bath and then centrifuged for 10 min at 5000 rpm in a JA10 rotor (Beckman) at 4°C. The cell pellets were resuspended in a total volume of 40 mls 1X binding buffer (40 mM imidazole, 0.5 M NaCl, 50 mM NaPO<sub>4</sub>, pH 8.0), transferred to two 50 ml Oakridge tubes and frozen at  
10 -70°C for at least 1 hr. The tubes were then thawed and the cells were lysed by sonication (using four successive 20 second bursts) on ice. The suspension was clarified by centrifugation 20-30 min at 9,000 rpm (10,000g) in a JA-17 rotor. The soluble lysate was batch absorbed to 7 ml of a 1:1 slurry of NiNTA resin:binding  
15 buffer by stirring 2-4 hr at 4°C. The slurry was centrifuged for 1 min at 500g in 50 ml tube (Falcon), resuspended in 5 mls binding buffer and poured into a 2.5 cm diameter column (BioRad). The column was attached to a UV monitor (ISCO) and the column was washed with binding buffer until a baseline was established. Imidazole was removed by washing with 50mM NaPO<sub>4</sub>, 0.3 M NaCl, 10% glycerol,  
20 pH 7.0 and bound protein was eluted using 50mM NaPO<sub>4</sub>, 0.3 M NaCl, 10% glycerol, pH 3.5-4.0.

The eluted proteins were stored at 4°C. Samples of total, soluble, and eluted proteins were resolved by SDS-PAGE. Protein samples were prepared for electrophoresis by mixing 1 $\mu$ l total (T) or soluble (S) protein with 4  $\mu$ l PBS and 5  $\mu$ l  
25 2X SDS-PAGE sample buffer, or 5  $\mu$ l eluted (E) protein and 5  $\mu$ l 2X SDS-PAGE sample buffer. The samples were heated to 95°C for 5 min, then cooled and 5 or 10  $\mu$ ls were loaded on 12.5% SDS-PAGE gels. Broad range molecular weight protein markers (BioRad) were also loaded to allow the MW of the identified fusion proteins to be estimated. After electrophoresis, protein was detected either generally by

staining gels with Coomassie blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein.

For Western blot analysis, the gels were blotted, and protein transfer was confirmed by Ponceau S staining as described in Example 22. After blocking the blots for 1 hr at room temperature in blocking buffer (PBST and 5% milk), 10 ml of a 1/500 dilution of an anti-*C. botulinum* toxin A IgY PEG prep (Ex. 3) in blocking buffer was added and the blots were incubated for an additional hour at room temperature. The blots were washed and developed using a rabbit anti-chicken alkaline phosphatase conjugate (Boehringer Mannheim) as the secondary antibody as described in Ex. 22. This analysis detected *C. botulinum* toxin A-reactive proteins in the pHisBotA (native and synthetic) protein samples (corresponding to the predicted full length proteins identified by Coomassie staining).

A gel containing proteins expressed from the pHisBot and pHisBot (native) constructs during various stages of purification and stained with Coomassie blue is shown in Figure 31. In Figure 31, lanes 1-4 and 9 contain proteins expressed by the pHisBotA construct (*i.e.*, the synthetic gene) and lanes 5-8 contain proteins expressed by the pHisBotA (native) construct. Lanes 1 and 5 contain total protein extracts; lanes 2 and 6 contain soluble protein extracts; lanes 3 and 7 contain proteins which flowed through the NiNTA columns; lanes 4, 8 and 9 contain protein eluted from the NiNTA columns and lane 10 contains molecular weight markers.

The above purification resulted in a yield of 3 mg (native gene) or 11 mg (synthetic gene) of affinity purified protein from a 1 liter starting culture, of which at least 90-95% of the protein was a single band of the predicted MW (50kd) and immunoreactivity for recombinant *C. botulinum* serotype A C fragment protein. Other than the level of expression, no difference was observed between the native and the synthetic gene expression systems.

These results demonstrate that soluble *C. botulinum* serotype A C fragment protein can be expressed in *E. coli* and purified utilizing either native or synthetic gene sequences.

## EXAMPLE 29

### Generation Of Neutralizing Antibodies Using A Recombinant *C. botulinum* Serotype A C Fragment Protein Containing A Six Residue His-Tag

5 In Example 27, neutralizing antibodies were generated utilizing the pHisBotA protein, which contains a histidine-tagged N-terminal extension comprising 10 histidine residues. To determine if the generation of neutralizing antibodies is dependent on the presence of this particular his-tag, a protein containing a shorter N-terminal extension (comprising 6 histidine residues) was produced and tested for the ability to generate neutralizing antibodies. This example involved a) the cloning and expression of the  
10 p6HisBotA(syn) protein and b) the generation and characterization of hyperimmune serum.

#### a) Cloning And Expression Of The p6HisBotA(syn) Protein

15 The p6HisBotA(syn) construct was generated as described below; the term "syn" designates the presence of synthetic gene sequences. This construct expresses the C fragment of the *C. botulinum* serotype A toxin with a histidine-tagged N terminal extension having the following sequence: MetHisHisHisHisHisHisMetAla (SEQ ID NO:32); the amino acids encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type.

20 6XHis oligonucleotides [5'-TATGCATCACCATCACCATCA-3' (SEQ ID NO:33) and 5'-CATGTGATGGTGATGGTGATGCA-3' (SEQ ID NO:34) were annealed as follows. One microgram of each oligonucleotide was mixed in total of 20  $\mu$ l 1X reaction buffer 2 (NEB) and the mixture was heated at 70°C for 5 min and then incubated at 42°C for 5 min. The annealed oligonucleotides were then ligated with gel  
25 purified *Nde*I/*Hind*III cleaved pET23b (T7 promoter) or pET21b (T7lac promoter) DNA and the gel purified *Nco*I/*Hind*III *C. botulinum* serotype A C fragment synthetic gene fragment derived from pAlterBot (Ex. 22). Recombinant clones were isolated and confirmed by restriction digestion. The DNA sequence encoding the 6X his-

tagged BotA protein contained within p6HisBotA(syn) is listed in SEQ ID NO:35. The amino acid sequence of the p6XHisBotA protein is listed in SEQ ID NO:36.

5 The resulting recombinant p6XHisBotA plasmid was transformed into the BL21(DE3) pLysS strain, and 1 liter cultures were grown, induced and harvested as described in Example 28. His-tagged protein was purified as described in Example 28, with the following modifications. The binding buffer (BB) contained 5 mM imidazole rather than 40 mM imidazole and NP40 was added to the soluble lysate to a final concentration of 0.1%. The bound material was washed on the column with BB until the baseline was established, then the column was washed successively with BB+20  
10 mM imidazole and BB+40 mM imidazole. The column was eluted as described in Example 28.

In the case of the pET23-derived expression system, high level expression of insoluble 6HisBotA protein was induced. The pET21-derived vector expressed lower levels of soluble protein that bound the NiNTA resin and eluted in the 40 mM  
15 imidazole wash rather than during the low pH elution. These results (*i.e.*, low level expression of a soluble protein) are consistent with the results obtained with pHisBotA protein (Ex. 25); the pHisBotA construct, like the pET21-derived vector, contains the T7lac rather than T7 promoter.

20 The 6HisBotA protein thus elutes under less stringent conditions than the 10X histidine-containing pHisBot protein (100-200 mM imidazole; Ex. 25) presumably due to the reduction in the length of the his-tag. The eluted protein was of the predicted size [*i.e.*, slightly reduced in comparison to pHisBotA protein].

#### **b) Generation And Characterization Of Hyperimmune Serum**

25 Eight BALBc mice were immunized with purified 6HisBotA protein using Gerbu GMDP adjuvant (CC Biotech). The 40 mM imidazole elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100  $\mu$ l antigen/adjuvant mix (12  $\mu$ g antigen + 1  $\mu$ g adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Control

mice received pHisBotB protein (prepared as described in Ex. 35 below) in Gerbu adjuvant.

5 Anti-*C. botulinum* serotype A toxoid titers were determined in serum from individual mice from each group using the ELISA described in Example 23a with the exception that the initial testing serum dilution was 1:100 in blocking buffer containing 0.5% Tween 20, followed by serial 5-fold dilutions into this buffer. The results of the ELISA demonstrated that seroconversion (relative to control mice) occurred in all 8 mice.

10 The ability of the anti-*C. botulinum* serotype A C fragment antibodies present in serum from the immunized mice to neutralize native *C. botulinum* type A toxin was tested using the mouse neutralization assay described in Example 23b. The amount of neutralizing antibodies present in the serum of the immunized mice was determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD<sub>50</sub> units of *C. botulinum* type A toxin and the mixtures were injected IP into  
15 mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum was found to protect 100% of the injected mice while the 1:10 diluted serum did not. This corresponds to a neutralization titer of 0.05-0.5 IU/ml.

20 These results demonstrate that neutralizing antibodies were induced when the 6HisBotA protein was utilized as the immunogen. Furthermore, these results demonstrate that seroconversion and the generation of neutralizing antibodies does not depend on the specific N terminal extension present on the recombinant *C. botulinum* type A C fragment proteins.



### EXAMPLE 30

#### Construction Of Vectors For The Expression Of His-Tagged *C. botulinum* Type A Toxin C Fragment Protein Using the Synthetic Gene

5 A number of expression vectors were constructed which contained the synthetic  
*C. botulinum* type A toxin C fragment gene. These constructs vary as to the promoter  
(T7 or T7lac) and repressor elements (lacIq) present on the plasmid. The T7 promoter  
is a stronger promoter than is the T7lac promoter. The various constructs provide  
varying expression levels and varying levels of plasmid stability. This example  
involved a) the construction of expression vectors containing the synthetic *C.*  
10 *botulinum* type A C fragment gene and b) the determination of the expression level  
achieved using plasmids containing either the kanamycin resistance or the ampicillin  
resistance genes in small scale cultures.

#### a) Construction Of Expression Vectors Containing The Synthetic *C. botulinum* Type A C Fragment Gene

15 Expression vectors containing the synthetic *C. botulinum* type A C fragment  
gene were engineered to utilize the kanamycin resistance rather than the ampicillin  
resistance gene. This was done for several reasons including concerns regarding the  
presence of residual ampicillin in recombinant protein derived from plasmids  
containing the ampicillin resistance gene. In addition, ampicillin resistant plasmids are  
20 more difficult to maintain in culture; the  $\beta$ -lactamase secreted by cells containing  
ampicillin resistant plasmids rapidly degrades extracellular ampicillin, allowing the  
growth of plasmid-negative cells.

A second altered feature of the expression vectors is the inclusion of lacIq gene  
in the plasmid. This repressor lowers expression from lac regulated promoters (the  
25 chromosomally located, lactose regulated T7 polymerase gene and the plasmid located  
T7lac promoter). This down regulates uninduced protein expression and can enhance  
the stability of recombinant cell lines. The final alteration to the vectors is the

inclusion of either the T7 or T7lac promoters that drive high or moderate level expression of recombinant protein, respectively.

5 The expression plasmids were constructed as follows. In all cases, the protein expressed is the pHisBotA(syn) protein previously described, and the only differences between constructs is the alteration of the various regulatory elements described above.

**i) Construction Of pHisBotA(syn) kan  
T7lac**

10 The pHisBotA(syn) kan T7lac construct was made by inserting the *SapI/XhoI* fragment containing the *C. botulinum* type A C fragment from pHisBotA(syn) into pET24 digested with *SapI/XhoI* (Novagen; fragment contains kan gene and origin of replication). The desired construct was selected for kanamycin resistance and confirmed by restriction digestion.

**ii) Construction Of pHisBotA(syn) kan  
lacIq T7lac**

15 The pHisBotA(syn) kan lacIq T7lac construct was made by inserting the *XbaI/HindIII* fragment containing the *C. botulinum* type A C fragment from pHisBotA(syn)kanT7lac into the pET24a vector digested with *XbaI/HindIII*. The resulting construct was confirmed by restriction digestion.

**iii) Construction Of pHisBotA(syn) kan  
lacIq T7**

20 The pHisBotA(syn) kan lacIq T7 construct was made by inserting the *XbaI/HindIII* fragment containing the *C. botulinum* type A C fragment from pHisBotA(syn) kan lacIq T7lac into *XbaI/HindIII*-digested pHisBotB(syn) kan lacIq T7 (described in Ex 37c below). The resulting construct was confirmed by restriction  
25 digestion.

**b) Determination Of The Expression Level Achieved  
Using Plasmids Containing Either The Kanamycin  
Resistance Or The Ampicillin Resistance Genes In  
Small Scale Cultures**

5 One liter cultures of pHisBotA(syn) kan T7lac/Bl21(DE3)pLysS and  
pHisBotA(syn) amp T7lac/Bl21(DE3)pLysS [this is the previously designated  
pHisBotA(syn) construct] were grown, induced and his-tagged proteins were purified  
as described in Example 28. No differences in yield or protein integrity/purity were  
observed.

10 These results demonstrate that the antigen induction levels from expression  
constructs were not affected by the choice of ampicillin versus kanamycin antibiotic  
resistance genes.

**EXAMPLE 31**

**Fermentation Of Cells Expressing Recombinant Botulinal Proteins**

15 **a) Fermentation Culture Of Cells Expressing  
Recombinant Botulinal Proteins**

Fermentation cultures were grown under the following conditions which were  
optimized for growth of the BL21(DE3) strains containing pET derived expression  
vectors. An overnight 1 liter feeder culture was prepared by inoculating of 1 liter  
20 media (in a 2L shaker flask) with a fresh colony grown on an LB kan plate. The  
feeder culture contained: 600 mls nitrogen source [20 gm yeast extract (BBL) and 40  
gm tryptone (BBL)/600 mls], 200 mls 5X fermentation salts (per liter: 48.5 gm  
K<sub>2</sub>HPO<sub>4</sub>, 12 gm NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O, 5 gm NH<sub>4</sub>Cl, 2.5 gm NaCl), 180 mls dH<sub>2</sub>O, 20 mls  
20% glucose, 2 mls 1 M MgSO<sub>4</sub>, 5 mls 0.05M CaCl<sub>2</sub> and 4 mls of a 10 mg/ml  
25 kanamycin stock. All solutions were sterilized by autoclaving, except the kanamycin  
stock which was filter sterilized.

An aliquot (5 ml) of the feeder culture broth was removed prior to inoculation, and grown for 2 days at 37°C as a culture broth sterility control. Growth was not observed in this control culture in any of the fermentations performed.

5 The inoculated feeder culture was grown for 12-15 hrs (ON) at 30-37°C. Care was taken to prevent oversaturation of this culture. The saturated feeder culture was added to 10L of fermentation media in fermenter (BiofloIV, New Brunswick Scientific, Edison, NJ) as follows. The fermenter was sterilized 120 min at 121°C with dH<sub>2</sub>O. The sterile water was removed, and fermentation media added as follows: 6 liters nitrogen source, 2 liters 5X fermentation salts, 2 liters 2% glucose, 20 mls 1 M  
10 MgSO<sub>4</sub>, 50 mls 0.05 M CaCl<sub>2</sub>, 2.5-3.5 mls Macol P 400 antifoam (PPG Industries Inc., Gurnee, IL), 40 mls 10mg/ml kanamycin and 10 mls trace elements (8 gm FeSO<sub>4</sub>•7H<sub>2</sub>O, 2 gm MnSO<sub>4</sub>•H<sub>2</sub>O, 2 gm AlCl<sub>3</sub>•6H<sub>2</sub>O, 0.8 gm CoCl<sub>2</sub>•6H<sub>2</sub>O, 0.4 gm ZnSO<sub>4</sub>•7H<sub>2</sub>O, 0.4 gm Na<sub>2</sub>MoO<sub>4</sub>•2H<sub>2</sub>O, 0.2 gm CuCl<sub>2</sub>•2H<sub>2</sub>O, 0.2 gm NiCl<sub>2</sub>, 0.1 gm H<sub>3</sub>BO<sub>4</sub>/200mls 5 M HCl). All solutions were sterilized by autoclaving, except the  
15 kanamycin stock which was filter sterilized. Fermentation media was prewarmed to 37°C before the addition of the feeder culture.

After the addition of the feeder culture, the culture was fermented at 37°C, 400 rpm agitation, and 10 l/min air sparging. The DO<sub>2</sub> control was set to 20% PID and dissolved oxygen levels were controlled by increasing the rate of agitation from  
20 400-850 rpm under DO<sub>2</sub> control. DO<sub>2</sub> levels were maintained at greater than or equal to 20% throughout the entire fermentation. When agitation levels reached 500-600 rpm the temperature was lowered to 30°C to reduce the oxygen consumption rate. Culture growth was continued until endogenous carbon sources were depleted. In these fermentations, glucose was depleted first [monitored with a glucose monitoring  
25 kit (Sigma)], followed by assimilation of acetate and other acidic carbons [monitored using an acetate test kit (Boehringer Mannheim)]. During the assimilation phase, the pH rose from 6.6-6.8 (starting pH) to 7.4-7.5, at which time the bulk of the remaining carbon source was depleted. This was signaled by a drop in agitation rate (from a maximum of 700-800 rpm) and a rise in DO<sub>2</sub> levels >30%. This corresponds to a  
30 OD<sub>600</sub> reading of 18-20/ml. At this point a fed batch mode was initiated, in which a

feed solution of 50% glucose was added at a rate of approximately 4 gm glucose/liter/hr. The pH was adjusted to 7.0 by the addition of 25%  $\text{H}_3\text{PO}_4$  (approximately 60 mls). Culture growth was continued and reached peak oxygen consumption within the next 3 hrs of growth (while the remaining residual non-glucose carbon sources were assimilated). This phase is characterized by a slow increase in pH, and air sparging was increased to 15L/min, to keep the maximum rpm below 850.

Once the residual acidic carbon sources are depleted the agitation rate decreases to 650-750 rpm and the pH begins to drop. pH control was maintained at 7.0 PID by regulated pump addition of a sterile 4M NaOH solution which was consumed at a steady rate for the remainder of the fermentation. Growth was continued at 30°C, and the cultures were grown linearly at a growth rate of 4-7  $\text{OD}_{600}$  units/hr, to at least 81.5  $\text{OD}_{600}$  units/ml (>30g/l dry cell weight) without induction. Antifoam (a 1:1 dilution with filter sterilized 100% ethanol) was added as necessary throughout the fermentation to prevent foaming.

During the fed batch mode, glucose was assimilated immediately (concentration in media consistently less than 0.1 gm/liter) and acetate was not produced in significant levels by the pET plasmid/BL21(DE3) cell lines tested (approximately 1 gm/liter at end of fermentation; this is lower than that observed in harvests from shaker flask cultures utilizing the same strains). This was fortuitous, since high levels of acetate has been shown to inhibit induction levels in a variety of expression systems. The above described conditions were found to be highly reproducible between fermentations and utilizing different expression plasmids. As a result, glucose and acetate level monitoring were no longer preformed during fermentation.

#### **b) Induction Of Fermentation Cultures**

Induction with IPTG (250 mg-10 gms, depending on the expression vector and experiment) was initiated 1-3 hrs after initiation of the glucose feed (30-50  $\text{OD}_{600}$ /ml). The growth rate after induction was monitored on a hourly basis. Aliquots (5-10 ml) of cells were harvested at the time of induction, and at hourly intervals post-induction. Optical density readings were determined by measuring the absorbance at 600 nm of

10  $\mu$ l culture in 990  $\mu$ l PBS versus a PBS control. The growth rate after induction was found to vary depending on the expression system utilized.

**c) Monitoring Of Fermentation Cultures**

Fermentation cultures were monitored using the following control assays.

5 **i) Colony Forming Ability**

An aliquots of cells were removed from the cultures at each timepoint sampled (uninduced and at various times after induction) were serially diluted in PBS (dilution 1=15  $\mu$ l cells/3 ml PBS, dilution 2 = 15  $\mu$ l of dilution 1/3 ml PBS, dilution 3 = 3 or 6  $\mu$ l of dilution 2/3mls PBS) and 100  $\mu$ l of dilution 3 was plated on an LB or TSA  
10 (trypticase soy agar) plate. The plates were incubated ON at 37°C and then the colonies are counted and scored for macro or micro growth.

**ii) Phenotypic Characterization**

Colonies growing on LB or TSA plates (above) from uninduced and induced timepoints were replica plated onto LB+kan, LB+chloramphenicol (for fermentations  
15 utilizing LysS or pACYCGro plasmids), LB+kan+1mM IPTG and LB plates, in this order. The plates were grown 6-8 hrs at 37°C and growth was scored on each plate for a minimum of 40-50 well isolated colonies. The percentage of cells retaining the plasmid at time of induction (*i.e.*, uninduced cultures immediately prior to the addition of IPTG) was determined to be the # colonies LB+Kan (or chloramphenicol) plate/#  
20 colonies LB plate X 100%. The percentage of cells with mutated pET plasmids was determined to be the # colonies LB+Kan+IPTG plate/# colonies LB plate X 100%. Colonies on all LB plates were scored morphologically for *E. coli* phenotype as a contamination control. Morphologically detectable contaminant colonies were not detected in any fermentation.

### iii) Recombinant BotA Protein Induction

A total of 10 OD<sub>600</sub> units of cells (*e.g.*, 200 µl of cells at OD<sub>600</sub>=50/ml) were removed from each timepoint sample to a 1.5 ml microfuge tube and pelleted for 2 min at maximum rpm in a microfuge. The pellets were resuspended in 1 ml of 50 mM NaHPO<sub>4</sub>, 0.5 M NaCl, 40mM imidazole buffer (pH 6.8) containing 1 mg/ml lysozyme. The samples were incubated for 20 min at room temperature and stored ON at -70°C. Samples were thawed completely at room temperature and sonicated 2 X 10 seconds with a Branson Sonifier 450 microtip probe at # 3 power setting. The samples were centrifuged for 5 min at maximum rpm in a microfuge.

An aliquot (20 µl) of the protein samples were removed to 20 µl 2X sample buffer, before or after centrifugation, for total and soluble protein extracts, respectively. The samples were heated to 95°C for 5 min, then cooled and 5 or 10 µl were loaded onto 12.5% SDS-PAGE gels. High molecular weight protein markers (BioRad) were also loaded to allow for estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining gels with Coomassie blue, or specifically, by blotting onto nitrocellulose (as described in Ex. 28) for Western blot detection of specific his-tagged proteins utilizing a NiNTA-alkaline phosphatase conjugate exactly as described by the manufacturer (Qiagen).

### iv) Recombinant Antigen Purification

At the end of each fermentation run, 1-10 liters of culture were harvested from the fermenter and the bacterial cells were pelleted by centrifugation at 6000 rpm for 10 min in a JA10 rotor (Beckman). The cell pellets were stored frozen at -70°C or utilized immediately without freezing. Cell pellets were resuspended to 15-20% weight to volume in resuspension buffer (generally 50 mM NaPO<sub>4</sub>, 0.5 M NaCl, 40mM imidazole, pH 6.8) and lysed utilizing either sonication or high pressure homogenization.

For sonication, the resuspension buffer was supplemented with lysozyme to 1 mg/ml, and the suspension was incubated for 20 min. at room temp. The sample was

then frozen ON at -70°C, thawed and sonicated 4 X 20 seconds at microtip maximum to reduce viscosity. For homogenization, the cells were lyzed by 2 passes through a homogenizer (Rannie Mini-lab type 8.30 H) at 600 Bar. Cell lysates were clarified by centrifugation for 30 min at 10,000 rpm in a JA10 rotor.

5 For IDA chromatography, samples were flocculated utilizing polyethyleneimine (PEI) prior to centrifugation. Cell pellets were resuspended in cell resuspension buffer (CRB: 50 mM NaPO<sub>4</sub>, 0.5 M NaCl, 40 mM imidazole, pH 6.8) to create a 20% cell suspension (wet weight of cells/volume of CRB) and cell lysates were prepared as described above (sonication or homogenization). PEI (a 2% solution in dH<sub>2</sub>O, pH 7.5  
10 with HCl) was added to the cell lysate a final concentration of 0.2%, and stirred for 20 min at room temperature prior to centrifugation (8,500 rpm in JA10 rotor for 30 minutes at 4°C). This treatment removed RNA, DNA and cell wall components, resulting in a clarified, low viscosity lysate ("PEI clarified lysate").

His-tagged proteins were purified from soluble lysates by metal-chelate affinity  
15 chromatography using either a NiNTA resin (as described in Ex. 28) or an IDA (iminodiacetic acid) resin as described below.

IDA resin affinity purifications were performed utilizing a low pressure chromatography system (ISCO). A 7 ml (small scale) or 70 ml (large scale) Chelating Sepharose Fast Flow (Pharmacia) affinity column was poured; in addition, a second  
20 guard column was poured and attached in line with the first column (to capture Ni ions that leached off the affinity column). The columns were washed with 3 column volumes of dH<sub>2</sub>O. The guard column was then removed and the affinity column was washed with 0.3 M NiSO<sub>4</sub> until resistivity was established, then with dH<sub>2</sub>O until the resistivity returned to baseline. The columns were reconnected and equilibrated with  
25 cell resuspension buffer (CRB; 50 mM NaPO<sub>4</sub>, 0.5 M NaCl, 40 mM imidazole, pH 6.8). The clarified sample (in CRB) was loaded. Flow rates were 5 ml/min for small scale columns and 20 ml/min for large scale columns. After sample loading, the column was washed with CRB until a baseline established and bound protein was eluted with elution buffer (50 mM NaPO<sub>4</sub>, 0.5 M NaCl, 800 mM imidazole, 20%  
30 glycerol, pH 6.8 or 8.0). Protein samples were stored at 4°C or -20°C. The yield of



eluted protein was established by measuring the OD<sub>280</sub> of the elutions, with a 1 mg/ml solution of protein assumed to yield an absorbance reading of 2.0.

The IDA columns may be regenerated and reused multiple times (>10). To regenerate the column, the column was washed with 2-3 column volumes of H<sub>2</sub>O, then 0.05 M EDTA until all of the blue/green color was removed followed by a wash with dH<sub>2</sub>O. The IDA columns were sterilized with 0.1 M NaOH (using at least 3 column volumes but not more than 50 minutes contact time with column packing material), then washed with 3 column volumes 0.05 M NaPO<sub>4</sub>, pH 5.0, then dH<sub>2</sub>O and stored at room temperature in 20 % ethanol.

### EXAMPLE 32

#### Construction Of A Folding Chaperone Overexpression System

Co-overexpression of the *E. coli* GroEL/GroES folding chaperones in a cell expressing a recombinant foreign protein has been reported to enhance the solubility of some foreign proteins that are otherwise insoluble when expressed in *E. coli* [Gragerouu *et al.* (1992) Proc. Natl. Acad. Sci. USA 89:10344]. The improvement in solubility is thought to be due to chaperone-mediated binding and unfolding of insoluble denatured proteins, thus allowing multiple attempts for productive refolding of recombinant proteins. By overexpressing the chaperones, the unfolding/refolding reaction is driven by excess chaperone, resulting, in some cases, in higher yields of soluble protein.

In this example, a chaperone overexpression system, compatible with pET vector expression systems, was constructed to facilitate testing chaperone-mediated solubilization of *C. botulinum* type A proteins. This example involved the cloning of the GroEL/ES operon and construction of a pLysS-based chaperone hyperexpression system.

The GroEL/GroES operon was PCR amplified and cloned into the pCRScript vector as described in Example 28. The following primer pair was used: 5'-CGCAT ATGAATATTCGTCCATTGCATG-3' (SEQ ID NO:37) [5' primer, start codon of

groES gene converted to *NdeI* site (underlined)] and 5'-GGAAGCTTGCAGGGCAAT  
TACATCATG (SEQ ID NO:38) (3' primer, stop codon of *groEL* gene italicized,  
engineered *HindIII* site underlined). Following amplification, the chaperone operon  
was excised as an *NdeI/HindIII* fragment and cloned into pET23b digested with *NdeI*  
5 and *HindIII*. This construction places the Gro operon under the control of the T7  
promoter of the pET23 vector. The desired construct was confirmed by restriction  
digestion.

The T7 promoter-Gro operon-T7 terminator expression cassette was then  
excised as a *BglII/BspEI* (filled) fragment and cloned into *BamHI* (compatible with  
10 *BglII/HindIII* (filled) cleaved pLysS plasmid (this removed the T7 lysozyme gene).  
The resulting construct was designated pACYCGro, since the plasmid utilizing the  
pACYC184 origin from the pLysS plasmid. Proper construction was confirmed by  
restriction digestion.

pACYCGro was transformed into BL21(DE3), cultures were grown and  
15 induced with 1 mM IPTG as described in preceding examples. Total and soluble  
protein extracts were generated from cells removed before and after IPTG induction  
and were resolved on a 12.5 % SDS-PAGE gel and stained with Coomassie blue.  
This analysis revealed that high levels of soluble GroEl and GroES proteins were made  
in the induced cells. These results demonstrated that the chaperone hyper-expression  
20 system was functional.

### EXAMPLE 33

#### Growth Of BotA/pACYCGro Cell Lines In Fermentation Cultures

Induction of BL21(DE3) cells lacking the LysS plasmid which contained BotA  
expression constructs grown in shaker flask or fermentation culture resulted in the  
25 expression of primarily insoluble BotA protein. Fermentation cultures were performed  
to determine if the simultaneous overexpression of the Gro operon and recombinant *C.*  
*botulinum* type A proteins (BotA proteins) resulted in enhanced solubility of the

recombinant BotA protein. This example involved the fermentation of  
pHisBotA(syn)kan lacIq T7lac/pACYCGro BL21(DE3) and pHisBotA(syn)kan lacIq  
T7/pACYCGro BL21(DE3) cell lines. The fermentations were repeated exactly as  
described in Example 31. Chloramphenicol (34 µg/ml) was included in the feeder and  
5 fermentation cultures.

a) **Fermentation Of pHisBotA(syn)kan lacIq  
T7lac/pACYCGro BL21(DE3) Cells**

For fermentation of cells containing plasmids comprising the T7lac promoter,  
induction was with 2 gms IPTG at 1 hr post initiation of glucose feed. The OD<sub>600</sub> was  
10 35 at time of induction, then 48.5, 61.5, 67 at 1-3 hrs post induction. Viable colony  
counts decreased from 0-3 hr induction [21 (13), 0, 0, 0; dilution 3 utilized 3 µl of  
dilution 2 cells] with numbers in parenthesis for the indicating microcolonies. Of 28  
colonies scored at the time of induction, 23 retained the pHisBotA(syn)kan lacIq T7lac  
plasmid (kan resistant), 22 contained the chaperone plasmid (chloramphenicol resistant)  
15 and no colonies at induction grew on IPTG+Kan plates (no mutations detected). These  
results were indicative of very strong promoter induction, since colony viability  
dropped immediately after induction.

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and  
stained with Coomassie. High level induction of Gro chaperones was observed, but  
20 very low level expression of soluble BotA protein was observed, increasing from 1 to  
4.0 hrs post induction (no expression detected in uninduced cells). The dramatically  
lower expression of the BotA antigen in the presence of chaperone may be due to  
promoter occlusion (*i.e.*, the stronger T7 promoter on the chaperone plasmid is  
preferentially utilized).

**b) Fermentation Of pHisBotA(syn)kan lacIq T7/  
pACYCGro BL21(DE3) Cells**

A fermentation utilizing the T7-driven BotA expression plasmid was performed. Induction was with 1 gm IPTG at 2 hrs post initiation of glucose feed. The OD<sub>600</sub> was 41 at time of induction, then 51.5, 61.5, 61.5 and 66 at 1-4 hrs post induction. Viable colony counts decreased from 0-4 hrs induction [71, 1 (34), 1 (1), 1, 0; dilution 3 utilized 6 µl dilution 2 cells) with numbers in parenthesis for the uninduced timepoint indicating microcolonies. Of 65 colonies scored at the time of induction, all 65 retained both the pHisBotA(syn)kan lacIq T7 plasmid (kan resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie. High level induction of Gro chaperones and moderate level expression of soluble BotA protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells).

A PEI-clarified lysate (0.2% final concentration PEI) [850 ml from 130 gm cell pellet (2 liters fermentation harvest)] was purified on a large scale IDA column. A total of 78 mg of protein was eluted. Extracts from the purification were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie. The elution was found to contain an approximately 1:1 mix of BotA/chaperone protein (Figure 32). PEI lysates prepared in this manner were typically 16 OD<sub>280</sub>/ml. This was estimated to be 8 mg protein/ml of lysate (by BCA assay). Thus, the eluted recombinant BotA protein represented 0.55% of the total soluble cellular protein applied to the column.

In Figure 32, lane 1 contains molecular weight markers, lanes 2-9 contain extracts from pHisBotA(syn)kan lacIq T7/pACYCGro/BL21(DE3) cells before or during purification on the IDA column. Lane 2 contains total protein extract; lane 3 contains soluble protein extract; lanes 4 and 5 contain PEI-clarified lysates (duplicates); lanes 6 and 7 contain flow-through from the IDA column (duplicates) and lanes 8 and 9 contain IDA column elute (lane 9 contains 1/10 the amount applied to lane 8).

These results demonstrate, that although the majority of the BotA protein produced was insoluble, 20 mg/liter of soluble recombinant BotA protein can be purified utilizing the pHisBotA(syn)kan lacIq T7/pACYCGro/BL21(DE3) expression system.

5

#### EXAMPLE 34

##### Purification Of Recombinant BotA Protein From Folding Chaperones

In this example of size exclusion chromatography was used to purify the recombinant BotA protein away from the folding chaperones and imidazole present in the IDA-purified material (Ex. 33).

10

To enhance the solubility of the recombinant BotA protein during scale-up, the protein was co-expressed with folding chaperones (Ex. 33). As observed with the recombinant BotB protein (Example 40 below), the folding chaperones co-eluted with the recombinant BotA protein during the Ni-IDA purification step. Because the recombinant BotA and BotB proteins have similar molecular weights (about 1/10 the size of the non-reduced folding chaperone) and the imidazole step gradient strategy was unsuccessful in purifying BotB away from the folding chaperone (see Ex. 40), size exclusion chromatography was examined for the ability to purify the recombinant BotA protein away from the folding chaperones.

15

A column (2.5 x 24 cm) containing Sephacryl S-100 HR (Pharmacia) was poured (bed volume ~ 110 ml). Proteins having molecular weights greater than 100 K are expected to elute in the void volume under these conditions and smaller proteins should be retained by the beads and elute at different times, depending on their molecular weights. To maintain solubility of the purified BotA protein, the Sephacryl column was equilibrated in a buffer having the same salt concentration as the buffer used to elute the BotA protein from the IDA column (*i.e.*, 50 mM sodium phosphate, 0.5 M NaCl, 10% glycerol; all reagents from Mallinkrodt, Chesterfield, MO).

20

25

Five milliliters of the IDA-purified recombinant BotA protein (Ex. 33) was filtered through a 0.45  $\mu$  syringe filter, applied to the column and the equilibration

buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm and collected either manually or with a fraction collector (BioRad). Appropriate fractions were pooled, if necessary, and the protein was quantitated by absorbance at 280 nm and/or BCA protein assay (Pierce).

5 The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the proteins present and to evaluate purity. The folding chaperone eluted first, followed by the recombinant BotA protein and then the imidazole peak.

SDS-PAGE analysis (12.5% polyacrylamide, reduced samples) was used to evaluate the purity of the IDA-purified recombinant BotA protein before and after  
10 S-100 purification. Figure 33 shows the difference in purity before and after the S-100 purification step. In Figure 33, lane 1 contains molecular weight markers (BioRad broad range). Lane 2 shows the IDA-purified recombinant BotA protein preparation, which is contaminated with significant amounts of the folding chaperone. Following S-100 purification, the amount of folding chaperone present in the BotA  
15 sample is reduced dramatically (lane 3). Lane 4 contains no protein (*i.e.*, it is a blank lane); lanes 5-8 contain samples of IDA-purified recombinant BotB and BotE proteins and are discussed *infra*.

Endotoxin levels in the S-100 purified BotA preparation were determined using the LAL assay (Associates of Cape Cod) as describe in Example 24. The purified  
20 BotA preparation was found to contain 22.7 to 45.5 EU/mg recombinant protein.

These results demonstrate that size exclusion chromatography was successful in purifying the recombinant BotA protein from folding chaperones and imidazole following an initial IDA purification step. Furthermore, these results demonstrate that the S-100 purified BotA protein was substantially free of endotoxin.

### EXAMPLE 35

#### Cloning And Expression Of The C Fragment Of The *C. botulinum* Serotype B Toxin Gene

5 The *C. botulinum* type B neurotoxin gene has been cloned and sequenced  
[Whelan *et al.* (1992) Appl. Environ. Microbiol. 58:2345 and Hutson *et al.* (1994)  
Curr. Microbiol. 28:101]. The nucleotide sequence of the toxin gene derived from the  
Eklund 17B strain (ATCC 25765) is available from the EMBL/GenBank sequence data  
banks under the accession number X71343; the nucleotide sequence of the coding  
region is listed in SEQ ID NO:39. The amino acid sequence of the *C. botulinum* type  
10 B neurotoxin derived from the strain Eklund 17B is listed in SEQ ID NO:40. The  
nucleotide sequence of the *C. botulinum* serotype B toxin gene derived from the  
Danish strain is listed in SEQ ID NO:41 and the corresponding amino acid sequence is  
listed in SEQ ID NO:42.

15 The DNA sequence encoding the native *C. botulinum* serotype B C fragment  
gene derived from the Eklund 17B strain can be expressed using the pETHisb vector;  
the resulting coding region is listed in SEQ ID NO:43 and the corresponding amino  
acid sequence is listed in SEQ ID NO:44. The DNA sequence encoding the native *C.*  
*botulinum* serotype B C fragment gene derived from the Danish strain can be  
expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID  
20 NO:45 and the corresponding amino acid sequence is listed in SEQ ID NO:46. The C  
fragment region from any strain of *C. botulinum* serotype B can be amplified and  
expressed using the approach illustrated below using the C fragment derived from *C.*  
*botulinum* type B 2017 strain.

25 The *C. botulinum* type B neurotoxin gene is synthesized as a single polypeptide  
chain which is processed to form a dimer composed of a light and a heavy chain  
linked via disulfide bonds; the type B neurotoxin has been reported to exist as a  
mixture of predominately single chain with some double chain (Whelan *et al.*, supra).  
The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C

fragment or the H<sub>C</sub> domain. Expression of the C fragment of *C. botulinum* type B toxin in heterologous hosts (e.g., *E. coli*) has not been previously reported.

The native C fragment of the *C. botulinum* serotype B toxin gene was cloned and expression constructs were made to facilitate protein expression in *E. coli*. This example involved PCR amplification of the gene, cloning, and construction of expression vectors.

The C fragment of the *C. botulinum* serotype B (BotB) toxin gene was cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. The *C. botulinum* type B 2017 strain was obtained from the American Type Culture Collection (ATCC #17843). The following primer pair was used to amplify the BotB gene: 5'-CGCCATGGCTGATACAATACTAATAGAA ATG-3' [5' primer, engineered *Nco*I site underlined (SEQ ID NO:47)] and 5'-GCAAG CTTTATTTCAGTCCACCCTTCATC-3' [3' primer, engineered *Hind*III site underlined, native gene termination codon italicized (SEQ ID NO:48)]. After cloning into the pCRscript vector, the *Nhe*I(filled)/*Hind*III fragment was cloned into pETHisB vector as described for BotA C fragment gene in Example 28. The resulting construct was termed pHisBotB.

pHisBotB expresses the BotB gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotB expression construct was transformed into BL21(DE3) pLysS competent cells and 1 liter cultures were grown, induced and his-tagged proteins were purified utilizing a NiNTA resin (eluted in low pH elution buffer) as described in Example 28. Total, soluble and purified proteins were resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a chicken anti-*C. botulinum* serotype B toxoid primary antibody (generated by immunization of hens using *C. botulinum* serotype B toxoid as described in Example 3). Samples of BotA and BotE C fragment proteins were included on the gels for MW and immunogenicity comparisons. Strong immunoreactivity to only the BotB protein was detected with the anti-*C. botulinum* serotype B toxoid antibodies. The



recombinant BotB protein was expressed at low levels (3 mg/liter) as a soluble protein. The purified BotB protein migrated as a single band of the predicted MW (i.e., ~50kD).

These results demonstrate the cloning of the native *C. botulinum* serotype B C fragment gene, the expression and purification of the recombinant BotB protein as a soluble his-tagged protein in *E. coli*.

### EXAMPLE 36

#### Generation Of Neutralizing Antibodies Using The Recombinant pHisBotB Protein

The ability of the purified pHisBot protein to generate neutralizing antibodies was examined. Nine BALBc mice were immunized with BotB protein (purified as described in Ex. 35) using Gerbu GMDP adjuvant (CC Biotech). The low pH elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100  $\mu$ l antigen/adjuvant mix (15  $\mu$ g antigen + 1  $\mu$ g adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Mice were subsequently boosted 1-2 weeks after bleeding and were then bled on day 70.

Anti-*C. botulinum* serotype B toxoid titers were determined in day 28 serum from individual mice from each group using the ELISA protocol outlined in Example 29 with the exception that the plates were coated with *C. botulinum* serotype B toxoid, and the primary antibody was a chicken anti-*C. botulinum* serotype B toxoid. Seroconversion [relative to control mice immunized with pHisBotE antigen (described below)] was observed with all 9 mice immunized with the purified pHisBotB protein.

The ability of the anti-BotB antibodies to neutralize native *C. botulinum* type B toxin was tested in a mouse-*C. botulinum* neutralization model using pooled mouse serum (see Ex. 23b). The LD<sub>50</sub> of purified *C. botulinum* type B toxin complex (Dr. Eric Johnson, University of Wisconsin, Madison) was determined by a intraperitoneal (IP) method [Schantz and Kautler (1978), *supra*] using 18-22 g female ICR mice. The amount of neutralizing antibodies present in the serum of the immunized mice was

determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD<sub>50</sub> units of *C. botulinum* type B toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum (day 28 or day 70) was found to protect 100% of the injected mice while the 1:10 diluted serum did not. This corresponds to a neutralization titer of 0.05-0.5 IU/ml.

These results demonstrate that seroconversion occurred and neutralizing antibodies were induced when the pHisBotB protein was utilized as the immunogen.

### EXAMPLE 37

#### Construction Of Vectors To Facilitate Expression Of His-Tagged BotB Protein In Fermentation Cultures

A number of expression vectors were constructed to facilitate the expression of recombinant BotB protein in large scale fermentation culture. These constructs varied as to the strength of the promoter utilized (T7 or T7lac) and the presence of repressor elements (lacIq) on the plasmid. The resulting constructs varied in the level of expression achieved and in plasmid stability which facilitated the selection of a optimal expression system for fermentation scaleup.

The BotB expression vectors created for fermentation culture were engineered to utilize the kanamycin rather than the ampicillin resistance gene, and contained either the T7 or T7lac promoter, with or without the lacIq gene for the reasons outlined in Example 30.

In all cases, the protein expressed by the various expression vectors is the pHisBot B protein described in Example 35, with the only differences between clones being the alteration of various regulatory elements. Using the designations outlined below, the pHisBotB clone (Ex. 35) is equivalent to pHisBotB amp T7lac.

**a) Construction Of pHisBotB kan T7lac**

pHisBotB kan T7lac was constructed by insertion of the *BglII/HindIII* fragment of pHisBotB which contains the BotB gene sequences into the pPA1870-2680 kan T7lac vector which had been digested with *BglII* and *HindIII* (the  
5 pPA1870-2680 kan T7lac vector contains the pET24 kan gene in the pET23 vector, such that no lacIq gene is present). Proper construction of pHisBotB kan T7lac was confirmed by restriction digestion.

**b) Construction Of pHisBotB kan lacIq T7lac**

pHisBotB kan lacIq T7lac was constructed by insertion of the *BglII/HindIII* fragment of pHisBotB which contains the BotB gene sequences into similarly cut  
10 pET24a vector. Proper construction of pHisBotB kan lacIq T7lac was confirmed by restriction digestion.

**c) Construction Of pHisBotB kan lacIq T7**

pHisBotB kan lacIq T7 was constructed by inserting the *NdeI/XhoI* fragment from pHisBotE kan lacIq T7lac which contains the BotB gene sequences into similarly  
15 cleaved pPA1870-2680 kan lacIq T7 vector (this vector contains the T7 promoter, the same N-terminal his-tag as the Bot constructs, the *C. difficile* toxin A insert, and the kan lacIq genes; this cloning replaces the *C. difficile* toxin A insert with the BotB insert). Proper construction was confirmed by restriction digestion.

20 Expression of recombinant BotB protein from these expression vectors and purification of the BotB protein is described in Example 38 below.

### EXAMPLE 38

Fermentation And Purification Of Recombinant BotB Protein Utilizing The  
pHisBotB kan lacIq T7lac, pHisBotB kan T7lac And pHisBotB kan lacIq T7 Vectors

5 The pHisBotB kan lacIq T7lac, pHisBotB kan T7lac and BotB kan lacIq T7  
constructs [all transformed into the BI21(DE3) strain] were grown in fermentation  
cultures to determine the utility of the various constructs for large scale expression and  
purification of soluble BotB protein. All fermentations were performed as described in  
Example 31.

10 **a) Fermentation Of pHisBotB kan lacIq T7lac/BI21(DE3)  
Cells**

The fermentation culture was induced 45 min post start of glucose feed with 1  
gm IPTG (final concentration = 0.4 mM). pH was maintained at 6.5 rather than 7.0.  
The OD<sub>600</sub> was 27 at time of induction, then 35, 38, and 40 at 1-3 hrs post induction.  
Duplicate platings of diluted 1 hr induction samples (dilutions were prepared as  
15 described Ex. 31, dilution 3 utilized 3 µl of dilution 2 cells) on TSA and LB+kan  
plates yielded 89 TSA colonies and 81 kan colonies (90% kan resistant).

Total and soluble protein extracts were resolved on a 12.5% SDS-PAGE gel  
and total protein was detected by staining with Coomassie blue. Low level induction  
of insoluble Bot B protein was observed, increasing from 1 to 3 hrs post induction (no  
20 expression was detected in uninduced cells).

**b) Fermentation Of pHisBotB kan T7lac/BI21(DE3) Cells**

The fermentation culture was induced 1 hr post start of glucose feed with 2 gm  
IPTG (final concentration = 0.8 mM). pH was maintained at 6.5 rather than 7.0. The  
OD<sub>600</sub> was 24.5 at time of induction, then 31.5, 32, and 33 at 1-3 hrs post induction,  
25 respectively. Duplicate platings of diluted 0 hr and 2 hr induction samples (dilutions  
were prepared as described Ex. 31; dilution 3 utilized 3 µl of dilution 2 cells) on TSA  
and LB+kan plates yielded 32 TSA colonies and 54 kan colonies (all kan resistant) for

uninduced cells, and 1 TSA colony and 0 kan colonies 2 hr post induction. These results were indicative of strong induction, since viable counts decreased dramatically 2 hrs post induction.

5 Total and soluble extracts were resolved on a 10% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. Moderate induction of insoluble BotB protein was observed, increasing from 1 to 3 hrs post induction (no expression was detected in uninduced cells).

**c) Fermentation Of pHisBotB kan lacIq T7/BI21(DE3)  
Cells**

10 The fermentation was induced 2 hr post start of glucose feed with 4 gm IPTG (final concentration = 1.6 mM). pH was maintained at 6.5 rather than 7.0. The OD<sub>600</sub> was 45 at time of induction, then 47, 50, and 50 and 55 at 1-4 hrs post induction, respectively. Viable colony counts decreased after induction (96, 1, 1, 2, 3; dilution 3 utilized 3 µl of dilution 2 cells). Of 63 colonies scored at the time of induction, all 63  
15 retaining the BotB plasmid (kan resistant) and no colonies at induction grew on IPTG + Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. Moderate level induction of insoluble BotB protein was observed, increasing from 1 to 4 hrs post induction (lower  
20 level expression was detected in uninduced cells, since the T7 rather than T7lac promoter was utilized).

**d) Purification Of pHisBotB Protein From pHisBotB  
amp T7lac/BI21(DE3) Cells**

25 Soluble recombinant BotB protein was purified utilizing NiNTA resin from 80 ml of cell lysate generated from cells harvested from a pHisBotB fermentation [using the pHisBotB amp T7lac/BI21(DE3) strain]. As predicted from the small scale results above, the majority of the induced protein was insoluble. As well, the eluted material was contaminated with multiple *E. coli* contaminant proteins. A Coomassie blue-

stained SDS-PAGE gel containing extracts derived from pHisBotB amp  
T7lac/BI21(DE3) cells before and during purification is shown in Figure 34. In Figure  
34, lane 1 contains broad range protein MW markers (BioRad). Lanes 2-5 contain  
extracts prepared from pHisBotB amp T7lac/BI21(DE3) cells grown in fermentation  
5 culture; lane 2 contains total protein; lane 3 contains soluble protein; lane 4 contains  
protein which did not bind to the NiNTA column (*i.e.*, the flow-through) and lane 5  
contains protein eluted from the NiNTA column.

Similar results were obtained using a small scale IDA column utilizing a cell  
lysate from the pHisBotB kan lacIq T7 fermentation described above. 250 mls of a  
10 20% w/v PEI clarified lysate (50 gms cell pellet) of botB kan lacIq T7/BI21(DE3)  
cells were purified on a small scale IDA column. The total yield of eluted protein was  
21 mg protein (assuming 1 mg/ml solution = 2 OD<sub>280</sub>/ml). When analyzed by  
SDS-PAGE and Coomassie staining, the BotB protein was found to comprise  
approximately 50% of the eluted protein with the remainder being a ladder of *E. coli*  
15 proteins similar to that observed with the NiNTA purification.

The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged  
proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble  
(after IDA column) and elution samples from the IDA column purification. The  
results demonstrated that a small percentage of BotB protein was soluble, that the  
20 soluble protein was not precipitated by PEI treatment and was quantitatively bound by  
the IDA column. Since a 1 liter fermentation harvest yielded a 67.5 gm cell pellet,  
this indicated that the yield of soluble affinity purified BotB protein from the IDA  
column was 14 mg/liter.

## EXAMPLE 39

### Co-Expression Of Recombinant BotB Proteins And Folding Chaperones In Fermentation Cultures

5 Fermentations were performed to determine if the simultaneous overexpression  
of folding chaperones (*i.e.*, the Gro operon) and the BotB protein resulted in enhanced  
solubility of the Bot B protein. This example involved fermentation of the  
pHisBotBkan lacIq T7lac/pACYCGro BL21(DE3), pHisBotB kan T7lac/pACYCGro  
BL21(DE3) and pHisBotBkan lacIq T7/ pACYCGro BL21(DE3) cell lines.  
10 Fermentation was carried out as described in Example 31; 34 µg/ml chloramphenicol  
was included in the feeder and fermentation cultures.

#### a) Fermentation Of pHisBotBkan lacIq T7lac/pACYCGro BL21(DE3) Cells

15 Induction was with 4 gms IPTG at 1 hr 15 min post initiation of the glucose  
feed. The OD<sub>600</sub> was 38 at time of induction, then 50, 58.5, 62 and 68 at 1-4 hrs post  
induction. Viable colony counts decreased during induction (24, 0, 0, 2, 0 at 0-4 hr  
induction; dilution 3 utilized 3 µl of dilution 2 cells). Of 24 colonies scored at the  
time of induction, 24 retained the BotB plasmid (kan resistant), 24 contained the  
chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on  
IPTG+Kan plates (no mutations detected).

20 Total and soluble extracts were resolved on 12.5% SDS-PAGE gels and were  
either stained with Coomassie blue or subjected to Western blotting (his-tagged  
proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate). This  
analysis revealed that the Gro chaperones were induced to high levels, but very low  
level expression of soluble BotB protein was observed, increasing from 1 to 4.0 hrs  
25 post induction (no expression detected in uninduced cells, induced protein detected  
only on Western blot). The dramatically lower expression of BotB protein in the  
presence of chaperone may be due to promoter occlusion (*i.e.*, the stronger T7  
promoter on the chaperone plasmid was preferentially utilized).

**b) Fermentation Of pHisBotB kan  
T7lac/pACYCGro/BI21(DE3) Cells**

Induction was with 4 gms IPTG at 1 hr post initiation of the glucose feed. The OD<sub>600</sub> was 33.5 at time of induction, then 44, 51, 58.5 and 69 at 1-4 hrs post  
5 induction. Viable colony counts decreased after 2 hrs induction (43, 65, 74, 0 (70), 0 (70) at 0-4 hr induction; bracketed numbers represent microcolonies; dilution 3 utilized 3 µl of dilution 2 cells). Most colonies at induction retained the BotB plasmid (kan resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

10 Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and subjected to Western blotting; his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate. This analysis revealed that the Gro chaperones were induced to high levels and low level expression of soluble Bot B protein was observed, increasing from 1 to 4.0 hrs post induction (no expression  
15 detected in uninduced cells).

A small scale IDA purification of BotB protein from a 250 ml PEI clarified 15% w/v extract (37.5 gm cell pellet) yielded approximately 12.5 mg protein, of which approximately 50% was BotB protein and 50% was GroEL chaperone (assessed by Coomassie staining of a 10% SDS-PAGE gel). The NiNTA alkaline phosphatase  
20 conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that all of the BotB protein produced by the pHisBotB kan T7lac/pACYCGro/BI21(DE3) cells was soluble; the BotB protein was not precipitated by PEI treatment and was quantitatively bound by  
25 the IDA column. Since a 1 liter fermentation harvest yielded a 75 gm cell pellet, this indicated that the yield of soluble affinity purified bot B protein from this fermentation was 12.5 mg/liter. These results also demonstrated that additional purification steps are necessary to separate the chaperone proteins from the BotB protein.



c) **Fermentation Of pHisBotBkan lacIq**

**T7/pACYCGro/BL21(DE3) Cells**

Induction was with 4 gms IPTG at 2 hr post initiation of the glucose feed. The OD<sub>600</sub> was 46 at time of induction, then 56, 63, 69 and 71.5 at 1-4 hrs post induction.

5     Viable colony counts decreased after induction (58, 3(5), 3, 0, 0 at 0-4 hr induction; bracketed numbers represent microcolonies; dilution 3 utilized 3 µl of dilution 2 cells). All (53/53) colonies scored at the time of induction retained the BotB plasmid (kan resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

10       Total and soluble extracts were resolved on a 10% SDS-PAGE gels and Western blotted and his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate. This analysis revealed that the Gro chaperones were induced to high levels (observed by ponceau S staining), and a much higher expression of soluble Bot B protein (compared to expression in the pHisBotB kan T7lac/pACYCGro  
15     fermentation) was observed at all timepoints, including uninduced cells (some increase in BotB protein levels were observed after induction).

A small scale IDA purification of BotB protein from a 100 ml PEI clarified 15% w/v extract (15 gm cell pellet) yielded approximately 40 mg protein, of which approximately 50% was BotB protein and 50% was GroEL chaperone, as assessed by  
20     Coomassie staining of a 10% SDS-PAGE gel. The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that a significant percentage (*i.e.*, ~10-20 %) of BotB protein was soluble, that the solubilized protein was not  
25     precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 10 liter fermentation yielded a 108 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from this fermentation was 144 mg/liter.

In a scale up experiment, 2 liters of a 20% w/v PEI clarified lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells were purified on a large scale  
30     IDA column. The purification was performed in duplicate. The total yield of BotB

protein was 220 and 325 mgs protein in the two experiments (assuming 1 mg/ml solution = 2.0 OD<sub>280</sub>/ml). This represents 0.7% or 1.0%, respectively, of the total soluble cellular protein (assuming a PEI lystate having a concentration of 8 mg protein/ml and that the eluted material comprises a 1:1 mixture of BotB and folding chaperone). The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. These results demonstrated that a significant percentage (*i.e.*, ~10-20 %) of the BotB protein was soluble, that the solubilized protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 108 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from the large scale purification was 60 mg or 89 mg/liter. These results also demonstrated that further purification would be necessary to remove the contaminating chaperone protein.

The above results provide methodologies for the purification of soluble BotB protein from fermentation cultures, in a form contaminated predominantly with a single *E. coli* protein (the folding chaperone utilized to enhance solubility). In the next example, methods are provided for the removal of the contaminating chaperone protein.

#### EXAMPLE 40

##### Removal Of Contaminating Folding Chaperone Protein From Purified Recombinant *C. botulinum* Type B Protein

In this example size exclusion chromatography and ultrafiltration was used to purify recombinant BotB protein from the folding chaperones and imidazole in IDA-purified material.

To enhance the solubility of the recombinant BotB protein during scale-up, the protein was co-expressed with folding chaperones (see Ex. 39). During the Ni-IDA purification step, the folding chaperones co-eluted with the BotB protein in 800 mM

imidazole; therefore, a second purification step was required to isolate the BotB free of folding chaperones. Lane 3 of Figure 35 contains proteins eluted from an IDA column to which a lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells had been applied; the proteins were resolved on a 4-15% polyacrylamide pre-cast gradient gel  
5 (Bio-Rad, Hercules, CA) run under native conditions and then stained with Coomassie blue. In Figure 35, lanes 1 and 4 contain proteins present in peak 1 and peak 2 from a Sephacryl S-100 column run as described below; lane 2 is blank.

As seen in lane 3 of Figure 35, the IDA-purified sample consists primarily of the folding chaperones and the BotB protein. The fact that the chaperones and the Bot  
10 B antigen appear as two distinct bands under native conditions suggested they were not complexed together and therefore, it should be possible to separate them, using either a gradient of imidazole concentrations or size exclusion methods.

In order to determine whether a gradient of imidazole concentrations could be used to separate the chaperone from the BotB protein, a step gradient using imidazole  
15 at 200, 400, 600, and 800 mM in 50 mM sodium phosphate, 0.5 M NaCl and 10 % glycerol, pH 6.8 was applied to an IDA column (containing proteins bound from a lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells). By narrowing the range of imidazole concentrations, it was hoped that the BotB and chaperone proteins would differentially elute at different concentrations of imidazole. Eluted proteins  
20 were monitored by absorbance at 280 nm and collected either manually or with a fraction collector (BioRad). Protein was found to elute at 200 and 400 mM imidazole only.

Figure 36 shows a Coomassie stained SDS-PAGE gel containing protein eluted during the imidazole step gradient. Lane 1 contains broad range MW markers  
25 (BioRad). Lane 2 contains BotB protein purified by IDA chromatography of an extract of pHisBotB/BL21(DE3) pLysS cells grown in shaker flask culture (*i.e.*, no co-expression of chaperones; Ex. 35). Lane 3 contains a 20% w/v PEI clarified lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells (*i.e.*, the lysate prior to purification by IDA chromatography). Lanes 4 and 5 contain protein which eluted at

200 or 400 mM imidazole, respectively. Lane 6 is blank. Lanes 7 and 8 contain 1/5 the load present in lanes 4 and 5.

As shown in Figure 36, both the chaperone and the BotB protein eluted in 200 mM imidazole, and more chaperone elutes in 400 mM imidazole, however no concentration of imidazole tested permitted the elution of BotB protein alone. Consequently, no significant purification was achieved using imidazole at these concentrations.

Because of the considerable difference in molecular weights between the folding chaperone, which is a multimer with a total molecular weight around 400 kD (as determined on a Shodex KB 804 sizing column by HPLC), and the recombinant BotB protein (molecular weight around 50 kD), size exclusion chromatography was next examined for the ability to separate these proteins.

#### a) Size Exclusion Chromatography

A column containing Sephacryl S-100 HR (S-100) (Pharmacia) was poured (2.5 cm x 24 cm ; ~110 ml bed volume). The column was equilibrated in a buffer consisting of phosphate buffered saline (10mM potassium phosphate, 150 mM NaCl, pH 7.2) and 10 % glycerol (Mallinkrodt). Typically, 5 ml of the IDA-purified BotB protein was filtered through a 0.45  $\mu$  syringe filter and applied to the column, and the equilibration buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm and collected either manually or with a fraction collector. Appropriate tubes were pooled, if necessary, and the protein was quantitated by absorbance at 280 nm and/or by BCA protein assay. The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the protein and evaluate the purity.

Because of its larger size, the folding chaperone eluted first, followed by the recombinant BotB protein. A smaller third peak was observed which failed to stain when analyzed by SDS-PAGE and therefore was presumed to be imidazole.

SDS-PAGE analysis (12.5% polyacrylamide, reduced samples) was used to evaluate the purity of the IDA-purified recombinant BotB protein before and after S-100 purification. The results are shown in Figure 33.

5 In Figure 33, lane 1 contains broad range MW markers (BioRad). Lane 5 contains IDA-purified BotB protein. Lane 6 contains IDA-purified BotB protein following S-100 purification. Lane 7 is blank (lanes 2-4 were discussed in Ex. 34 above).

10 The results shown in Figure 33 show that the IDA-purified BotB is significantly contaminated with the folding chaperone (molecular weight about 60 kD under reducing conditions; lane 6). Following S-100 purification, the amount of folding chaperone present in the BotB sample was reduced dramatically (lane 7). Visual inspection of the Coomassie stained SDS-PAGE gel revealed that after S-100 purification, > 90% of the total protein present was BotB.

15 The IDA-purified BotB and the S-100-purified BotB samples were analyzed by HPLC on a size exclusion column (Shodex KB 804); this analysis revealed that the BotB protein represented 64% of the total protein in the IDA-purified sample and that following S-100 purification, the BotB protein represented >95% of the total protein in the sample.

20 The IDA-purified BotB material was also applied to a ACA 44 (SpectraPor, Houston, TX) column. The ACA 44 resin is equivalent to the S-100 resin and chromatography using the ACA 44 resin was carried out exactly as described above for the S-100 resin. The ACA 44 resin was found to separate the recombinant BotB protein from the folding chaperone. The ACA 44-purified BotB sample was analyzed for endotoxin using the LAL assay (Associates of Cape Cod) as describe in Example 25 24. Two aliquots of the ACA 44-purified BotB preparation were analyzed and were found to contain either 58 to 116 EU/mg recombinant protein or 94 to 189 EU/mg recombinant protein.

30 These results demonstrate that size exclusion chromatography can be used to purify the recombinant BotB protein from the folding chaperone and imidazole in IDA-purified material.

**b) Ultrafiltration For The Separation Of Recombinant BotB Protein And Chaperones**

Ultrafiltration was examined as an alternative method for the separation recombinant BotB protein and folding chaperones in IDA-purified material. While in this example only mixtures of BotB and chaperones were separated by ultrafiltration, this technique is suitable for use with recombinant BotA and BotE proteins as well provided that the wash buffers used are altered as necessary to take into account different requirements for solubility.

The recombinant BotB protein and folding chaperones were separated using a two-step sequential ultrafiltration method. The first membrane used had a nominal molecular weight cutoff (MWCO) of approximately 100 kD; this membrane retains the larger folding chaperone while allowing the smaller recombinant protein to pass through. The addition of several volumes of wash buffer may be required to efficiently wash the recombinant protein through the membrane. The second step utilized a membrane with a nominal MWCO of approximately 10 kD. During this step, the recombinant antigen was retained by the membrane and could be concentrated to the degree desired and the imidazole and excess wash buffer passed through the membrane.

Twenty-seven milliliters of an IDA-purified BotB preparation was ultrafiltered through a 47 mm YM 100 (100 kD MWCO) membrane (Amicon) in a 50 ml stirred cell (Amicon). The membrane was washed in dd H<sub>2</sub>O prior to use as recommended by the manufacturer. Six volumes of 10% glycerol in PBS were washed through to remove most of the recombinant BotB protein and this wash was collected in a separate vessel. The resulting BotB protein-rich filtrate was then concentrated 12-fold using a YM 10 (10 kD MWCO) membrane (Amicon), to a final volume of 14 ml. The YM 100 and YM 10 concentrates were analyzed along with the lysate starting material by native PAGE using a 4 -15% pre-cast gradient gel (BioRad). The results are shown in Figure 37.

In Figure 37, lane 1 contains IDA-purified BotB derived from a shaker flask culture (*i.e.*, no co-expression of chaperones; Ex. 35); lane 2 contains a 20% w/v PEI

clarified lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells; lane 3 shows the lysate of lane 3 after IDA purification; lane 4 contains the YM 10 concentrate and lane 5 contains the YM 100 concentrate.

5 The results shown in Figure 37 demonstrate that the recombinant BotB protein can be purified away from the folding chaperone by ultrafiltration through a 100 kD MWCO membrane (lane 4), leaving the chaperone protein in the 100 kD concentrate (lane 5). Analysis of the sample in lane 5 also showed that very little of the BotB protein was retained by the 100 kD MWCO membrane after 6 volumes of wash buffer had been applied.

10 The BotB samples following IDA chromatography and following ultrafiltration through the YM 100 membrane were analyzed by HPLC on a size exclusion column (Shodex KB 804); this analysis revealed that the BotB protein represented 64% of the total protein in the IDA-purified sample and that following ultrafiltration through the YM 100 membrane, the BotB protein represented >96% of the total protein in the  
15 sample.

The BotB protein purified by ultrafiltration through the YM 100 membrane was examined for endotoxin using the LAL assay (Associates of Cape Cod) as describe in Example 24. Two aliquots of the YM 100-purified BotB preparation were analyzed and were found to contain either 18 to 36 EU/mg recombinant protein or 125 to 250  
20 EU/mg recombinant protein.

The above results demonstrate that size exclusion chromatography and ultrafiltration can be used to purify recombinant botulinal toxin proteins away from folding chaperones.

## EXAMPLE 41

### Cloning And Expression Of The C Fragment Of The *C. botulinum* Serotype E Toxin Gene

5 The *C. botulinum* type E neurotoxin gene has been cloned and sequenced from  
several different strains [Poulet *et al.* (1992) Biochem. Biophys. Res. Commun.  
183:107 (strain Beluga); Whelan *et al.* (1992) Eur. J. Biochem. 204:657 (strain NCTC  
11219); Fujii *et al.* (1990) Microbiol. Immunol. 34:1041 (partial sequence of strains  
Mashike, Iwani and Otaru) and Fujii *et al.* (1993) J. Gen. Microbiol. 139:79 (strain  
Mashike)]. The nucleotide sequence of the type E toxin gene is available from the  
10 EMBL sequence data bank under accession numbers X62089 (strain Beluga) and  
X62683 (strain NCTC 11219). The nucleotide sequence of the coding region (strain  
Beluga) is listed in SEQ ID NO:49. The amino acid sequence of the *C. botulinum*  
type E neurotoxin derived from strain Belgua is listed in SEQ ID NO:50. The  
nucleotide sequence of the coding region (strain NCTC 11219) is listed in SEQ ID  
15 NO:51. The amino acid sequence of the *C. botulinum* type E neurotoxin derived from  
strain NCTC 11219 is listed in SEQ ID NO:52.

The DNA sequence encoding the native *C. botulinum* serotype E C fragment  
gene derived from the Beluga strain can be expressed as a histidine-tagged protein  
using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:53 and  
20 the corresponding amino acid sequence is listed in SEQ ID NO:54. The DNA  
sequence encoding the C fragment of the native *C. botulinum* serotype E gene derived  
from the NCTC 11219 strain can be expressed as a histidine-tagged fusion protein  
using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:55 and  
the corresponding amino acid sequence is listed in SEQ ID NO:56. The C fragment  
25 region from any strain of *C. botulinum* serotype E can be amplified and expressed  
using the approach illustrated below using the C fragment derived from *C. botulinum*  
type E 2231strain (ATCC #17786).



The type E neurotoxin gene is synthesized as a single polypeptide chain which may be converted to a double-chain form (*i.e.*, a heavy chain and a light chain) by cleavage with trypsin; unlike the type A neurotoxin, the type E neurotoxin exists essentially only in the single-chain form. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H<sub>C</sub> domain. Expression of the C fragment of *C. botulinum* type E toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

The native C fragment of the *C. botulinum* serotype E toxin (BotE) gene was cloned and inserted into expression vectors to facilitate expression of the recombinant BotE protein in *E. coli*. This example involved PCR amplification of the gene, cloning, and construction of expression vectors.

The BotE serotype gene was isolated using PCR as described for the BotA serotype gene in Example 28. The *C. botulinum* type E strain was obtained from the American Type Culture Collection (ATCC #17786; strain 2231). The following primer pair was used in the PCR amplification: 5'-CGCCATGGCTCTTTCTTCTTATACAGATGAT-3' (5' primer, engineered *Nco*I site underlined) (SEQ ID NO:57) and 5'-GCAAGCTTTTATTTTCTTGCCATCCATG-3' (3' primer, engineered *Hind*III site underlined, native gene termination codon italicized) (SEQ ID NO:58). The PCR product was inserted into pCRscript as described in Example 28. The resulting pCRscript BotE clone was confirmed by restriction digestion, as well as, by obtaining the sequence of approximately 300 bases located at the 5' end of the C fragment coding region using standard DNA sequencing methods. The resulting BotE sequence was identical to that of the published *C. botulinum* type E toxin sequence [Whelan *et al* (1992), *supra*].

The *Nhe*I(filled)/*Hind*III fragment from a pCRscript BotE recombinant was cloned into pETHisb vector as described for BotA C fragment in Example 28. The resulting construct was termed pHisBotE. pHisBotE expresses the BotE gene under the control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag.

The pHisBotE expression construct was transformed into BL21(DE3) pLysS competent cells and 1 liter cultures were grown, induced and his-tagged proteins were purified utilizing a NiNTA resin (eluted in low pH elution buffer) as described in Example 28. Total, soluble and purified proteins were resolved by SDS-PAGE and  
5 detected by Coomassie staining. The results are shown in Figure 38.

In Figure 38, lane 1 contains broad range MW markers (BioRad); lane 2 contains a total protein extract; lane 3 contains a soluble protein extract; lane 4 contains proteins present in the flow through from the NiNTA column (this sample was not diluted prior to loading and therefore represents a load 5X that of the load  
10 applied for the total and soluble extracts in lanes 2 and 3); lane 5 contains proteins eluted from the NiNTA column; lane 6 contains protein eluted from a NiNTA column which had been stored at -20°C for 1 year.

The pHisBotE protein was expressed at moderate levels (7 mg/liter) as a totally soluble protein. The purified protein migrated as a single band of the predicted MW.

15 Western blot hybridization utilizing a chicken anti-*C. botulinum* serotype E toxoid primary antibody (generated by immunization of hens as described in Example 3 using *C. botulinum* serotype E toxoid ) was also performed on the total, soluble and purified BotE proteins. Samples of BotA and BotB C fragments were also included on the gels to facilitate MW and immunogenicity comparisons. Strong immunoreactivity  
20 was detected using the anti-*C. botulinum* type E toxoid antibody only with the BotE protein.

These results demonstrate that the native BotE gene sequences can be expressed as a soluble his-tagged protein in *E. coli* and purified by metal-chelation affinity chromatography.

## EXAMPLE 42

### Generation Of Neutralizing Antibodies Using The Recombinant pHisBotE Protein

The ability of the purified pHisBotE protein to generate neutralizing antibodies was examined. Nine BALBc mice were immunized with BotE protein (purified as described in Ex. 41) using Gerbu GMDP adjuvant (CC Biotech). The low pH elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100  $\mu$ l antigen/adjuvant mix (35  $\mu$ g antigen + 1  $\mu$ g adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Mice were subsequently boosted and bled on day 70.

Anti-*C. botulinum* serotype E toxoid titers were determined in day 28 serum from individual mice from each group using the ELISA protocol outlined in Example 29 with the exception that the plates were coated with *C. botulinum* serotype E toxoid, and the primary antibody was a chicken anti-*C. botulinum* serotype E toxoid. Seroconversion [relative to control mice immunized with the p6xHisBotA antigen (Ex. 29)] was observed with all 9 mice immunized with the purified pHisBotE protein.

The ability of the anti-BotE antibodies to neutralize native *C. botulinum* type E toxin was tested in a mouse-*C. botulinum* neutralization model using pooled mouse serum (see Ex. 23b). The LD<sub>50</sub> of purified *C. botulinum* type E toxin complex (Dr. Eric Johnson, University of Wisconsin, Madison) was determined by a intraperitoneal (IP) method [Schantz and Kautler (1978), supra] using 18-22 g female ICR mice. The amount of neutralizing antibodies present in the serum of the immunized mice was determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD<sub>50</sub> units of *C. botulinum* type E toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum from day 28 did not protect, while undiluted, 1/10 diluted and 1/100 diluted day 70 serum protected (100% of animals) while 1/1000 diluted day 70 serum did not. This corresponds to a neutralization titer of 50-500 IU/ml.

These results demonstrate that seroconversion occurred and neutralizing antibodies were induced when the recombinant BotE protein was utilized as the immunogen.

### EXAMPLE 43

#### 5                                      Construction Of Vectors To Facilitate Expression                                     Of His-Tagged BotE Protein In Fermentation Cultures

10                                      A number of expression vectors were constructed to facilitate the expression of recombinant BotE protein in large scale fermentation culture. These constructs varied as to the strength of the promoter utilized (T7 or T7lac) and the presence of repressor elements (lacIq) on the plasmid. The resulting constructs varied in the level of expression achieved and in plasmid stability which facilitated the selection of a optimal expression system for fermentation scaleup. This example involved a) construction of BotE expression vectors and b) determination of expression levels in small scale cultures.

#### 15                                      a)        **Construction Of BotE Expression Vectors**

                                    The BotE expression vectors created for fermentation culture were engineered to utilize the kanamycin rather than the ampicillin resistance gene, and contained either the T7 or T7lac promoter, with or without the lacIq gene for the reasons outlined in Example 30.

20                                      In all cases, the protein expressed by the various expression vectors is the pHisBotE protein described in Example 41, with the only differences between clones being the alteration of various regulatory elements. Using the designations outlined below, the pHisBotE clone (Ex. 41) is equivalent to pHisBotE amp T7lac.

**i) Construction Of pHisBotE kan lacIq  
T7lac**

pHisBotE kan lacIq T7lac was constructed by inserting the *XbaI/HindIII* fragment of pHisBotE which contains the BotE gene sequences into *XbaI/HindIII*-cleaved pET24a vector. Proper construction was confirmed by restriction digestion.

**ii) Construction Of pHisBotE kan T7**

pHisBotE kan T7 was constructed by ligating the BotE-containing *XbaI/SapI* fragment of pHisBotE kan lacIqT7lac to the T7 promoter-containing *XbaI/SapI* fragment of pET23a. Proper construction was confirmed by restriction digestion.

**iii) Construction Of pHisBotE kan lacIqT7**

pHisBotE kan lacIqT7 was constructed by inserting the *BglII/HindIII* fragment from pHisBotE kan T7 which contains the BotE gene sequences into *BglII/HindIII*-cleaved pET24 vector. Proper construction was confirmed by restriction digestion.

**b) Determination Of BotE Expression Levels In Small  
Scale Cultures**

The three BotE kan expression vectors described above were transformed into B121(DE3) competent cells and 50 ml (2XYT + 40 µg/ml kan) cultures were grown and induced with IPTG as described in Example 28. Total and soluble protein extracts from before and after induction made as described in Example 28. The total and soluble extracts were resolved on a 12.5% SDS-PAGE gel, and his-tagged proteins were detected on a Western blot utilizing the NiNTA-alkaline phosphatase conjugate as described in Example 31(c)(iii). The results showed that all three BotE cell lines expressed his-tagged proteins of the predicted MW for the BotE protein upon induction. The results also demonstrated that the two constructs that contained the T7 promoter expressed the BotE protein before induction, while the T7lac promoter construct did not. Upon induction, the T7 promoter-containing constructs induced to

higher levels than the T7lac-containing construct, with the pHisBotE kan lacIq T7/BI21(DE3) cells accumulating the maximal levels of BotE protein.

#### EXAMPLE 44

##### Expression And Purification Of pHisBotE From Fermentation Cultures

5           Based on the small scale inductions performed in Example 43, the pHisBotE kan lacIq T7/BI21(DE3) strain was selected for fermentation scaleup. This example involved the fermentation and purification of recombinant BotE C fragment protein.

          A fermentation with the pHisBotE kan lacIq T7/BI21(DE3) strain was performed as described in Example 31. The fermentation culture was induced 2 hrs  
10       post start of the glucose feed with 4 gm IPTG (final concentration = 1.6 mM). The OD<sub>600</sub> was 42 at time of induction, then 46.5, 48, 53 and 54 at 1-4 hrs post induction. Viable colony counts decreased from 0-4 hr induction [131, 4 (28), 7 (3), 7, 8; dilution 3 utilized 6 µl of dilution 2 cells; bracketed colonies are microcolonies]. All (32/32) colonies scored at the time of induction retained the BotE plasmid (kan resistant) and  
15       no colonies at induction grew on IPTG+Kan plates (no mutations detected). These results were indicative of strong promoter induction, since colony viability reduced after induction, and the culture stopped growing during fermentation (stopped at 54 OD<sub>600</sub>/ml).

          Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and total  
20       protein was detected by staining with Coomassie blue. The results are shown in Figure 39.

          In Figure 39, lane 1 contains total protein from a pHisBotA kan T7 lac/BI21(DE3) pLysS fermentation (Ex. 24). Lanes 2-9 contain extracts prepared from the above pHisBotE kan lacIq T7/BI21(DE3) fermentation; lanes 2- 4 contain total  
25       protein extracts prepared at 0, 1 and 2 hours post-induction, respectively. Lane 5 contains a soluble protein extract prepared at 2 hours post-induction. Lanes 6 and 7 contain total and soluble extracts prepared at 3 hours post-induction, respectively.

Lanes 8 and 9 contain total and soluble extracts prepared at 4 hours post-induction, respectively. Lane 10 contains broad range MW markers (BioRad).

5 The results shown in Figure 39 demonstrate that moderate level induction of totally soluble Bot E protein was observed, increasing from 1 to 4 hrs post induction (no expression was detected in uninduced cells). From a 2 liter fermentation harvest a 155 gm (wet wt) cell pellet was obtained and used to make a PEI-clarified lysate (1 liter in CRB, pH 6.8). The lysate was applied to a large scale IDA column and 200 mg of BotE protein, which was found to be greater than 95% pure (as judged by visual inspection of a Coomassie stained SDS-PAGE gel), was recovered. This  
10 represents 2.5% of the total soluble cellular protein (assuming a PEI lysate having a concentration of 8 mg protein/ml) and corresponds to a yield of 100 mg BotE protein/liter of fermentation culture.

The above results demonstrate that high levels of the recombinant BotE protein can be expressed and purified from fermentation cultures.

15

#### EXAMPLE 45

##### Removal Of Imidazole From Purified Recombinant BotE Protein Preparations

The expression of recombinant BotE protein, unlike the BotA and BotB proteins, did not require the presence of folding chaperones to maintain solubility during scale-up. A size exclusion chromatography step was included however to  
20 remove the imidazole from the sample and exchange the IDA elution buffer for one consistent with the BotA antigen.

A Sephacryl S-100 HR (S-100; Pharmacia) column was poured (2.5 cm x 24 cm; bed volume ~ 110 ml). Under these conditions, the BotE protein should be retained by the beads to a lesser degree than the smaller imidazole, therefore the BotE  
25 protein should elute from the column before the imidazole. The column was equilibrated in a buffer consisting of 50 mM sodium phosphate, 0.5 M NaCl, and 10% glycerol (all reagents from Mallinkrodt). Five milliliters of the IDA-purified BotE protein (Ex. 44) was filtered through a 0.45  $\mu$  syringe filter and applied to the S-100

column, and equilibration buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm, and collected either manually or with a fraction collector. Appropriate tubes were pooled if necessary, and the protein was quantitated by absorbance at 280 nm and/or BCA protein assay. The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the protein(s) and evaluate the purity.

Figure 40 provides a representative chromatogram showing the purification of IDA-purified BotE on the S-100 column. Even though folding chaperones were not over-expressed with this antigen, a small amount of protein eluted at a time consistent with the folding chaperones expressed with BotA and BotB proteins (Gro) (see the first peak). The second peak in the chromatogram contained the BotE protein, and the third peak was presumably imidazole. This presumed imidazole peak was isolated in comparable levels in IDA-purified BotA and BotB protein preparations as well.

These results demonstrate that size exclusion chromatography can be used to remove imidazole and traces of contaminating high molecular weight proteins from IDA-purified BotE protein preparations.

The S-100-purified BotE protein was tested for endotoxin contamination using the LAL assay as described in Example 24. This preparation was found to contain 64 to 128 EU/mg recombinant protein and is therefore substantially free of endotoxin.

The S-100 purified BotE was mixed with purified preparations of BotA and BotB proteins and used to immunize mice; 5  $\mu$ g of each Bot protein was used per immunization and alum was included as an adjuvant. After two immunizations with this trivalent vaccine, the immunized mice were challenged with *C. botulinum* toxin. The immunized mice contained neutralizing antibodies sufficient to neutralize between 100,000 to 1,000,000 LD<sub>50</sub> of either toxin A or toxin B and between 1,000 to 10,000 LD<sub>50</sub> of toxin E. The titer of neutralizing antibodies directed against toxin E would be expected to increase following subsequent boosts with the vaccine. These results demonstrate that a trivalent vaccine containing recombinant BotA, BotB and BotE proteins provokes neutralizing antibodies.



## EXAMPLE 46

### Expression Of The C Fragment Of The *C. botulinum* Serotype C Toxin Gene And Generation Of Neutralizing Antibodies

5 The *C. botulinum* type C1 neurotoxin gene has been cloned and sequenced  
[Kimura *et al.* (1990) Biochem. Biophys. Res. Comm. 171:1304]. The nucleotide  
sequence of the toxin gene derived from the *C. botulinum* type C strain C-Stockholm  
is available from the EMBL/GenBank sequence data banks under the accession number  
D90210; the nucleotide sequence of the coding region is listed in SEQ ID NO:59. The  
amino acid sequence of the *C. botulinum* type C1 neurotoxin derived from this strain  
10 is listed in SEQ ID NO:60.

The DNA sequence encoding the native *C. botulinum* serotype C1 C fragment  
gene derived from the C-Stockholm strain can be expressed using the pETHisb vector;  
the resulting coding region is listed in SEQ ID NO:61 and the corresponding amino  
acid sequence is listed in SEQ ID NO:62. The C fragment region from any strain of  
15 *C. botulinum* serotype C can be amplified and expressed using the approach illustrated  
below using the C fragment derived from *C. botulinum* type C C-Stockholm strain.  
Expression of the C fragment of *C. botulinum* type C1 toxin in heterologous hosts  
(*e.g.*, *E. coli*) has not been previously reported.

The C fragment of the *C. botulinum* serotype C1 (BotC1) toxin gene is cloned  
20 using the protocols and conditions described in Example 28 for the isolation of the  
native BotA gene. A number of *C. botulinum* serotype C strains (expressing either or  
both C1 and C2 toxin) are available from the ATCC [*e.g.*, 2220 (ATCC 17782), 2239  
(ATCC 17783), 2223 (ATCC 17784; a type C- $\beta$  strain; C- $\beta$  strains produce C2 toxin),  
662 (ATCC 17849; a type C- $\alpha$  strain; C- $\alpha$  strains produce mainly C1 toxin and a  
25 small amount of C2 toxin), 2021 (ATCC 17850; a type C- $\alpha$  strain) and VPI 3803  
(ATCC 25766)]. Alternatively, other type C strains may be employed for the isolation  
of sequences encoding the C fragment of *C. botulinum* serotype C toxin.

The following primer pair is used to amplify the BotC gene: 5'-CGCCATGGC  
TTTATTAAAAGATATAATTAATG-3' [5' primer, engineered *Nco*I site underlined  
(SEQ ID NO:63)] and 5'-GCAAGCTTTTATTCACTTACAGGTAC AAAACC-3' [3'  
primer, engineered *Hind*III site underlined, native gene termination codon italicized  
(SEQ ID NO:64)]. Following PCR amplification, the PCR product is inserted into the  
pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as  
described for BotA C fragment gene in Example 28. The resulting construct is termed  
pHisBotC. Proper construction is confirmed by DNA sequencing of the BotC  
sequences contained within pHisBotC.

pHisBotC expresses the BotC gene sequences under the transcriptional control  
of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag  
affinity tag. The pHisBotC expression construct is transformed into BL21(DE3)  
pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins  
are purified utilizing a NiNTA resin (eluted in 250 mM imidazole, 20% glycerol) as  
described in Example 28. Total, soluble and purified proteins are resolved by  
SDS-PAGE and detected by Coomassie staining and Western blot hybridization  
utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes  
his-tagged proteins as described in Example 31(c)(iii). This analysis permits the  
determination of expression levels of the pHisBotC protein (*i.e.*, number of mg/liter  
expressed as a soluble protein). The purified BotC protein will migrate as a single  
band of the predicted MW (*i.e.*, ~50kD).

The level of expression of the pHisBotC protein may be modified (increased)  
by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the *lacIq*  
gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in  
fermentation cultures as described in Example 30. If only very low levels (*i.e.*, less  
than 0.5%) of soluble pHisBotC protein are expressed using the above expression  
systems, the pHisBotC construct may be co-expressed with pACYCGro construct as  
described in Example 32. In this case, the recombinant BotC protein may co-purify  
with the folding chaperones. The contaminating chaperones may be removed as

described in Example 34. Preparations of purified pHisBotC protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotC protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotC protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotC antibodies to neutralize native *C. botulinum* type C toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

#### EXAMPLE 47

##### Expression Of The C Fragment Of The *C. botulinum*

##### Serotype D Toxin Gene And Generation Of Neutralizing Antibodies

The *C. botulinum* type D neurotoxin gene has been cloned and sequenced [Sunagawa et al. (1992) J. Vet. Med. Sci. 54:905 and Binz *et al.* (1990) Nucleic Acids Res. 18:5556]. The nucleotide sequence of the toxin gene derived from the CB16 strain is available from the EMBL/GenBank sequence data banks under the accession number S49407; the nucleotide sequence of the coding region is listed in SEQ ID NO:65. The amino acid sequence of the *C. botulinum* type D neurotoxin derived from the CB16 strain is listed in SEQ ID NO:66.

The DNA sequence encoding the native *C. botulinum* serotype D C fragment gene derived from a BotD expressing strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:67 and the corresponding amino acid sequence is listed in SEQ ID NO:68. The C fragment region from any strain of *C. botulinum* serotype D can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type D CB16 strain. Expression of the C fragment of *C. botulinum* type D toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

The C fragment of the *C. botulinum* serotype D (BotD) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the

native BotA gene. A number of *C. botulinum* type D strains are available from the ATCC [e.g., ATCC 9633, 2023 (ATCC 17851), and VPI 5995 (ATCC 27517)].

The following primer pair is used to amplify the BotD gene: 5'-CGCCATGGC  
5 TTTATTAAAAGATATAATTAATG-3' [5' primer, engineered *Nco*I site underlined  
(SEQ ID NO:63)] and 5'-GCAAGCTTTTACTCTACCCATCCTGGATCCCT-3' [3'  
primer, engineered *Hind*III site underlined, native gene termination codon italicized  
(SEQ ID NO:69)]. Following PCR amplification, the PCR product is inserted into the  
pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as  
described for BotA C fragment gene in Example 28. The resulting construct is termed  
10 pHisBotD.

pHisBotD expresses the BotD gene sequences under the transcriptional control  
of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag  
affinity tag. The pHisBotD expression construct is transformed into BL21(DE3)  
pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins  
15 are purified utilizing a NiNTA resin as described in Example 28. Total, soluble and  
purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and  
Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen)  
which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis  
permits the determination of expression levels of the pHisBotD protein (*i.e.*, number of  
20 mg/liter expressed as a soluble protein). The purified BotD protein will migrate as a  
single band of the predicted MW (*i.e.*, ~50kD).

The level of expression of the pHisBotD protein may be modified (increased)  
by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the *lacIq*  
gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in  
25 fermentation cultures as described in Example 30. If only very low levels (*i.e.*, less  
than about 0.5%) of soluble pHisBotD protein are expressed using the above  
expression systems, the pHisBotD construct may be co-expressed with pACYCGro  
construct as described in Example 32. In this case, the recombinant BotD protein may  
co-purify with the folding chaperones. The contaminating chaperones may be removed

as described in Example 34. Preparations of purified pHisBotD protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotD protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotD protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotD antibodies to neutralize native *C. botulinum* type D toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

#### EXAMPLE 48

##### Expression Of The C Fragment Of The *C. botulinum*

##### 10 Serotype F Toxin Gene And Generation Of Neutralizing Antibodies

The *C. botulinum* type F neurotoxin gene has been cloned and sequenced [East *et al.* (1992) FEMS Microbiol. Lett. 96:225]. The nucleotide sequence of the toxin gene derived from the 202F strain (ATCC 23387) is available from the EMBL/GenBank sequence data banks under the accession number M92906; the nucleotide sequence of the coding region is listed in SEQ ID NO:70. The amino acid sequence of the *C. botulinum* type F neurotoxin derived from the 202F strain is listed in SEQ ID NO:71.

The DNA sequence encoding the native *C. botulinum* serotype F C fragment gene derived from the 202F strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:72 and the corresponding amino acid sequence is listed in SEQ ID NO:73. The C fragment region from any strain of *C. botulinum* serotype F can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type F 202F strain. Expression of the C fragment of *C. botulinum* type F toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

The C fragment of the *C. botulinum* serotype F (BotF) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the

native BotA gene. The *C. botulinum* type F 202F strain is obtained from the American Type Culture Collection (ATCC 23387). Alternatively, sequences encoding the BotF toxin may be isolated from any BotF expressing strain [e.g., VPI 4404 (ATCC 25764), VPI 2382 (ATCC 27321) and Langeland (ATCC 35415)].

5           The following primer pair is used to amplify the BotF gene: 5'-CGCCATGGC  
TATTCTAATTATATATTTTAATAG-3' [5' primer, engineered *Nco*I site underlined  
(SEQ ID NO:74)] and 5'-GCAAGCTTTCATTCTTCCATCCATTCTC-3' [3' primer,  
engineered *Hind*III site underlined, native gene termination codon italicized (SEQ ID  
NO:75)]. Following PCR amplification, the PCR product is inserted into the  
10 pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as  
described for BotA C fragment gene in Example 28. The resulting construct is termed  
pHisBotF.

pHisBotF expresses the BotF gene sequences under the transcriptional control  
of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag  
15 affinity tag. The pHisBotF expression construct is transformed into BL21(DE3) pLysS  
competent cells and 1 liter cultures are grown, induced and his-tagged proteins are  
purified utilizing a NiNTA resin as described in Example 28. Total, soluble and  
purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and  
Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen)  
20 which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis  
permits the determination of expression levels of the pHisBotF protein (*i.e.*, number of  
mg/liter expressed as a soluble protein). The purified BotF protein will migrate as a  
single band of the predicted MW (*i.e.*, ~50kD).

The level of expression of the pHisBotF protein may be modified (increased)  
25 by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the *lacIq*  
gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in  
fermentation cultures as described in Example 30. If only very low levels (*i.e.*, less  
than about 0.5%) of soluble pHisBotF protein are expressed using the above expression  
systems, the pHisBotF construct may be co-expressed with pACYCGro construct as

described in Example 32. In this case, the recombinant BotF protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotF protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

5           The purified pHisBotF protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotF protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotF antibodies to neutralize native *C. botulinum* type F toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

10

#### EXAMPLE 49

##### Expression Of The C Fragment Of The *C. botulinum* Serotype G Toxin Gene And Generation Of Neutralizing Antibodies

15

The *C. botulinum* type G neurotoxin gene has been cloned and sequenced [Campbell *et al.* (1993) *Biochimica et Biophysica Acta* 1216:487 and Binz *et al.* (1990) *Nucleic Acids Res.* 18:5556]. The nucleotide sequence of the toxin gene derived from the 113/30 strain (NCFB 3012) is available from the EMBL/GenBank sequence data banks under the accession number X74162; the nucleotide sequence of the coding region is listed in SEQ ID NO:76. The amino acid sequence of the *C. botulinum* type G neurotoxin derived from this strain is listed in SEQ ID NO:77.

20

The DNA sequence encoding the native *C. botulinum* serotype G C fragment gene derived from the 113/30 strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:78 and the corresponding amino acid sequence is listed in SEQ ID NO:79. The C fragment region from any strain of *C. botulinum* serotype G can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type G 113/30 strain. Expression of the C fragment of *C. botulinum* type G toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

25

The C fragment of the *C. botulinum* serotype G (BotG) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. The *C. botulinum* type G 113/30 strain is obtained from the NCFB. The following primer pair is used to amplify the BotG gene: 5'-CGCCATGGCTGAC  
5 ACAATTTTAATACA AGT-3' [5' primer, engineered *Nco*I site underlined (SEQ ID NO:80)] and 5'-GCCTCGAGTTATTCTGTCCATCCTTCATCCAC-3' [3' primer, engineered *Xho*I site underlined, native gene termination codon italicized (SEQ ID NO:81)]. Following PCR amplification, the PCR product is inserted into the pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as  
10 described for BotA C fragment gene in Example 28 with the exception that the sequences encoding BotG are excised from the pCRscript vector by digestion with *Nco*I and *Xho*I and the *Nco*I site is blunted (the BotG sequences contain an internal *Hind*III site). This *Nco*I(filled)/*Xho*I fragment is then ligated to the pETHisb vector which has been digested with *Nhe*I and *Sal*I and the *Nhe*I site is blunted. The  
15 resulting construct is termed pHisBotG.

pHisBotG expresses the BotG gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotG expression construct is transformed into BL21(DE3) pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins  
20 are purified utilizing a NiNTA resin as described in Example 28. Total, soluble and purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis permits the determination of expression levels of the pHisBotG protein (*i.e.*, number of  
25 mg/liter expressed as a soluble protein). The purified BotG protein will migrate as a single band of the predicted MW (*i.e.*, ~50kD).

The level of expression of the pHisBotG protein may be modified (increased) by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the lacIq gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in



fermentation cultures as described in Example 30. If only very low levels (*i.e.*, less than about 0.5%) of soluble pHisBotG protein are expressed using the above expression systems, the pHisBotG construct may be co-expressed with pACYCGro construct as described in Example 32. In this case, the recombinant BotG protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotG protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotG protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotG protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotG antibodies to neutralize native *C. botulinum* type G toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

## EXAMPLE 50

### Expression Of Recombinant Botulinal Toxin Proteins In Eucaryotic Host Cells

Recombinant botulinal C fragment proteins may be expressed in eucaryotic host cells, such as yeast and insect cells.

#### a) Expression In Yeast

Botulinal C fragments derived from serotypes A, B, C, D, E, F and G may be expressed in yeast cells using a variety of commercially available vectors. For example, the pPIC3K and pPIC9K expression vectors (Invitrogen) may be employed for expression in the methylotrophic yeast, *Pichia pastoris*. When the pPIC3K vector is employed, expression of the botulinal C fragment protein will be intracellular. When the pPIC9K vector is employed, the botulinal C fragment protein will be secreted (the alpha factor secretion signal is provided on the pPIC9K vector).

DNA sequences encoding the desired C fragment is inserted into these vectors using techniques known to the art. Briefly, the desired botulinal expression cassette (including sequences encoding the his-tag; described in the preceding examples) is

amplified using the PCR in conjunction with primers that incorporate unique restriction sites at the termini of the amplified fragment. Suitable restriction enzyme sites include *Sna*BI, *Eco*RI, *Avr*II and *Not*I. When the botulinal C fragment is to be expressed using the pPIC3K vector, the initiator methionine (ATG) is provided by the desired Bot gene sequence and a Kozak consensus sequence is engineered upstream of the ATG (*e.g.*, ACCATGG).

The amplified restriction fragment containing the botulinal C fragment gene is then cloned into the desired expression vector. Recombinant clones are integrated into the *Pichia pastoris* genome and recombinant protein expression is induced using methanol following the manufacturer's instructions (Invitrogen *Pichia* expression kit manual).

*C. botulinum* genes are A/T rich and contain multiple sequences that are similar to yeast transcriptional termination signals (*e.g.*, TTTTATA). If premature transcription termination is observed when the botulinal C fragment genes are expressed in yeast, the transcription termination signals present in the C fragment genes can be removed by either site directed mutagenesis (utilizing the pALTER system; Promega) or by construction of synthetic genes utilizing overlapping synthetic primers.

The botulinal C fragment genes may be expressed in other yeast cells using other commercially available vectors [*e.g.*, using the pYES2 vector (Invitrogen) and *S. cerevisiae* cells (Invitrogen)].

#### **b) Expression In Insect Cells**

Botulinal C fragments derived from serotypes A, B, C, D, E, F and G may be expressed in insect cells using a variety of commercially available vectors. For example, the pBlueBac4 transfer vector (Invitrogen) may be employed for expression in *Spodoptera frugiperda* (*Sf9*) insect cells (baculovirus expression system) (equivalent baculovirus vectors and host cells are available from other vendors, *e.g.*, Pharmingen, San Diego, CA). Botulinal C fragments contained on *Nco*I/*Hind*III fragments

contained within the pHisBotA-G expression constructs (described in the preceding examples) are cloned into the pBlueBac4 vector (digested with *NcoI* and *HindIII*); the *NcoI* site present on the C fragment constructs overlaps with the start codon of the fusion proteins. In the case of botulinal C fragment clones that contain internal  
5 *HindIII* sites (e.g., using the BotG sequences described in Ex. 49), the C fragment gene is contained within a *NcoI/XhoI* fragment on the pHisBot construct. This *NcoI/XhoI* fragment is excised from pHisBot and inserted into pBlueBac4 digested with *NcoI* and *SaII*. Recombinant baculoviruses are made and the desired recombinant C fragment is expressed in *Sf9* cells using the protocols provided by the manufacturer  
10 (Invitrogen MaxBac manual). The resulting constructs will express the pHisBot protein intracellularly (including the N-terminal his-tag) under the control of the polyhedrin promoter. For extracellular secretion of botulinal C fragment proteins, the C fragment sequences from the pHisBot constructs are cloned into the pMelBacB vector (Invitrogen) as described above for the pBlueBac4 vector. When the pMelBacB  
15 vector is employed, the his-tagged botulinal C fragment proteins are secreted (utilizing a vector-encoded honeybee melittin secretion signal) and contain a nine amino acid extension at the N-terminus.

His-tagged botulinal C fragments expressed in yeast or insect cells are purified using metal chelation columns as described in the preceding examples.

20 From the above it is clear that the present invention provides compositions and methods for the preparation of effective multivalent vaccines against *C. botulinum* neurotoxin. It is also contemplated that the recombinant botulinal proteins be used for the production of antitoxins. All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and  
25 variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Williams, James A.  
Thalley, Bruce S.
- (ii) TITLE OF INVENTION: Multivalent Vaccine For Clostridium  
Botulinum Neurotoxin
- (iii) NUMBER OF SEQUENCES: 82
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Medlen & Carroll
  - (B) STREET: 220 Montgomery Street, Suite 2200
  - (C) CITY: San Francisco
  - (D) STATE: California
  - (E) COUNTRY: United States of America
  - (F) ZIP: 94104
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Ingolia, Diane E.
  - (B) REGISTRATION NUMBER: 40,027
  - (C) REFERENCE/DOCKET NUMBER: OPHD-02304
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (415) 705-8410
  - (B) TELEFAX: (415) 397-8338

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGAAATTTAG CTGCAGCATC TGAC

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCTAGCAAAT TCGCTTGTGT TGAA

24

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTCGCATATA GCATTAGACC

20

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTATCTAGGC CTAAAGTAT

19

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 8133 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..8130

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG TCT TTA ATA TCT AAA GAA GAG TTA ATA AAA CTC GCA TAT AGC ATT  
Met Ser Leu Ile Ser Lys Glu Glu Leu Ile Lys Leu Ala Tyr Ser Ile  
1 5 10 15

48

AGA CCA AGA GAA AAT GAG TAT AAA ACT ATA CTA ACT AAT TTA GAC GAA Arg Pro Arg Glu Asn Glu Tyr Lys Thr Ile Leu Thr Asn Leu Asp Glu 20 25 30	96
TAT AAT AAG TTA ACT ACA AAC AAT AAT GAA AAT AAA TAT TTG CAA TTA Tyr Asn Lys Leu Thr Thr Asn Asn Asn Glu Asn Lys Tyr Leu Gln Leu 35 40 45	144
AAA AAA CTA AAT GAA TCA ATT GAT GTT TTT ATG AAT AAA TAT AAA ACT Lys Lys Leu Asn Glu Ser Ile Asp Val Phe Met Asn Lys Tyr Lys Thr 50 55 60	192
TCA AGC AGA AAT AGA GCA CTC TCT AAT CTA AAA AAA GAT ATA TTA AAA Ser Ser Arg Asn Arg Ala Leu Ser Asn Leu Lys Lys Asp Ile Leu Lys 65 70 75 80	240
GAA GTA ATT CTT ATT AAA AAT TCC AAT ACA AGC CCT GTA GAA AAA AAT Glu Val Ile Leu Ile Lys Asn Ser Asn Thr Ser Pro Val Glu Lys Asn 85 90 95	288
TTA CAT TTT GTA TGG ATA GGT GGA GAA GTC AGT GAT ATT GCT CTT GAA Leu His Phe Val Trp Ile Gly Gly Glu Val Ser Asp Ile Ala Leu Glu 100 105 110	336
TAC ATA AAA CAA TGG GCT GAT ATT AAT GCA GAA TAT AAT ATT AAA CTG Tyr Ile Lys Gln Trp Ala Asp Ile Asn Ala Glu Tyr Asn Ile Lys Leu 115 120 125	384
TGG TAT GAT AGT GAA GCA TTC TTA GTA AAT ACA CTA AAA AAG GCT ATA Trp Tyr Asp Ser Glu Ala Phe Leu Val Asn Thr Leu Lys Lys Ala Ile 130 135 140	432
GTT GAA TCT TCT ACC ACT GAA GCA TTA CAG CTA CTA GAG GAA GAG ATT Val Glu Ser Ser Thr Thr Glu Ala Leu Gln Leu Leu Glu Glu Glu Ile 145 150 155 160	480
CAA AAT CCT CAA TTT GAT AAT ATG AAA TTT TAC AAA AAA AGG ATG GAA Gln Asn Pro Gln Phe Asp Asn Met Lys Phe Tyr Lys Lys Arg Met Glu 165 170 175	528
TTT ATA TAT GAT AGA CAA AAA AGG TTT ATA AAT TAT TAT AAA TCT CAA Phe Ile Tyr Asp Arg Gln Lys Arg Phe Ile Asn Tyr Tyr Lys Ser Gln 180 185 190	576
ATC AAT AAA CCT ACA GTA CCT ACA ATA GAT GAT ATT ATA AAG TCT CAT Ile Asn Lys Pro Thr Val Pro Thr Ile Asp Asp Ile Ile Lys Ser His 195 200 205	624
CTA GTA TCT GAA TAT AAT AGA GAT GAA ACT GTA TTA GAA TCA TAT AGA Leu Val Ser Glu Tyr Asn Arg Asp Glu Thr Val Leu Glu Ser Tyr Arg 210 215 220	672
ACA AAT TCT TTG AGA AAA ATA AAT AGT AAT CAT GGG ATA GAT ATC AGG Thr Asn Ser Leu Arg Lys Ile Asn Ser Asn His Gly Ile Asp Ile Arg 225 230 235 240	720
GCT AAT AGT TTG TTT ACA GAA CAA GAG TTA TTA AAT ATT TAT AGT CAG Ala Asn Ser Leu Phe Thr Glu Gln Glu Leu Leu Asn Ile Tyr Ser Gln 245 250 255	768

GAG TTG TTA AAT CGT GGA AAT TTA GCT GCA GCA TCT GAC ATA GTA AGA Glu Leu Leu Asn Arg Gly Asn Leu Ala Ala Ala Ser Asp Ile Val Arg 260 265 270	816
TTA TTA GCC CTA AAA AAT TTT GGC GGA GTA TAT TTA GAT GTT GAT ATG Leu Leu Ala Leu Lys Asn Phe Gly Gly Val Tyr Leu Asp Val Asp Met 275 280 285	864
CTT CCA GGT ATT CAC TCT GAT TTA TTT AAA ACA ATA TCT AGA CCT AGC Leu Pro Gly Ile His Ser Asp Leu Phe Lys Thr Ile Ser Arg Pro Ser 290 295 300	912
TCT ATT GGA CTA GAC CGT TGG GAA ATG ATA AAA TTA GAG GCT ATT ATG Ser Ile Gly Leu Asp Arg Trp Glu Met Ile Lys Leu Glu Ala Ile Met 305 310 315 320	960
AAG TAT AAA AAA TAT ATA AAT AAT TAT ACA TCA GAA AAC TTT GAT AAA Lys Tyr Lys Lys Tyr Ile Asn Asn Tyr Thr Ser Glu Asn Phe Asp Lys 325 330 335	1008
CTT GAT CAA CAA TTA AAA GAT AAT TTT AAA CTC ATT ATA GAA AGT AAA Leu Asp Gln Gln Leu Lys Asp Asn Phe Lys Leu Ile Ile Glu Ser Lys 340 345 350	1056
AGT GAA AAA TCT GAG ATA TTT TCT AAA TTA GAA AAT TTA AAT GTA TCT Ser Glu Lys Ser Glu Ile Phe Ser Lys Leu Glu Asn Leu Asn Val Ser 355 360 365	1104
GAT CTT GAA ATT AAA ATA GCT TTC GCT TTA GGC AGT GTT ATA AAT CAA Asp Leu Glu Ile Lys Ile Ala Phe Ala Leu Gly Ser Val Ile Asn Gln 370 375 380	1152
GCC TTG ATA TCA AAA CAA GGT TCA TAT CTT ACT AAC CTA GTA ATA GAA Ala Leu Ile Ser Lys Gln Gly Ser Tyr Leu Thr Asn Leu Val Ile Glu 385 390 395 400	1200
CAA GTA AAA AAT AGA TAT CAA TTT TTA AAC CAA CAC CTT AAC CCA GCC Gln Val Lys Asn Arg Tyr Gln Phe Leu Asn Gln His Leu Asn Pro Ala 405 410 415	1248
ATA GAG TCT GAT AAT AAC TTC ACA GAT ACT ACT AAA ATT TTT CAT GAT Ile Glu Ser Asp Asn Asn Phe Thr Asp Thr Thr Lys Ile Phe His Asp 420 425 430	1296
TCA TTA TTT AAT TCA GCT ACC GCA GAA AAC TCT ATG TTT TTA ACA AAA Ser Leu Phe Asn Ser Ala Thr Ala Glu Asn Ser Met Phe Leu Thr Lys 435 440 445	1344
ATA GCA CCA TAC TTA CAA GTA GGT TTT ATG CCA GAA GCT CGC TCC ACA Ile Ala Pro Tyr Leu Gln Val Gly Phe Met Pro Glu Ala Arg Ser Thr 450 455 460	1392
ATA AGT TTA AGT GGT CCA GGA GCT TAT GCG TCA GCT TAC TAT GAT TTC Ile Ser Leu Ser Gly Pro Gly Ala Tyr Ala Ser Ala Tyr Tyr Asp Phe 465 470 475 480	1440
ATA AAT TTA CAA GAA AAT ACT ATA GAA AAA ACT TTA AAA GCA TCA GAT Ile Asn Leu Gln Glu Asn Thr Ile Glu Lys Thr Leu Lys Ala Ser Asp 485 490 495	1488

TTA ATA GAA TTT AAA TTC CCA GAA AAT AAT CTA TCT CAA TTG ACA GAA Leu Ile Glu Phe Lys Phe Pro Glu Asn Asn Leu Ser Gln Leu Thr Glu 500 505 510	1536
CAA GAA ATA AAT AGT CTA TGG AGC TTT GAT CAA GCA AGT GCA AAA TAT Gln Glu Ile Asn Ser Leu Trp Ser Phe Asp Gln Ala Ser Ala Lys Tyr 515 520 525	1584
CAA TTT GAG AAA TAT GTA AGA GAT TAT ACT GGT GGA TCT CTT TCT GAA Gln Phe Glu Lys Tyr Val Arg Asp Tyr Thr Gly Gly Ser Leu Ser Glu 530 535 540	1632
GAC AAT GGG GTA GAC TTT AAT AAA AAT ACT GCC CTC GAC AAA AAC TAT Asp Asn Gly Val Asp Phe Asn Lys Asn Thr Ala Leu Asp Lys Asn Tyr 545 550 555 560	1680
TTA TTA AAT AAT AAA ATT CCA TCA AAC AAT GTA GAA GAA GCT GGA AGT Leu Leu Asn Asn Lys Ile Pro Ser Asn Asn Val Glu Glu Ala Gly Ser 565 570 575	1728
AAA AAT TAT GTT CAT TAT ATC ATA CAG TTA CAA GGA GAT GAT ATA AGT Lys Asn Tyr Val His Tyr Ile Ile Gln Leu Gln Gly Asp Asp Ile Ser 580 585 590	1776
TAT GAA GCA ACA TGC AAT TTA TTT TCT AAA AAT CCT AAA AAT AGT ATT Tyr Glu Ala Thr Cys Asn Leu Phe Ser Lys Asn Pro Lys Asn Ser Ile 595 600 605	1824
ATT ATA CAA CGA AAT ATG AAT GAA AGT GCA AAA AGC TAC TTT TTA AGT Ile Ile Gln Arg Asn Met Asn Glu Ser Ala Lys Ser Tyr Phe Leu Ser 610 615 620	1872
GAT GAT GGA GAA TCT ATT TTA GAA TTA AAT AAA TAT AGG ATA CCT GAA Asp Asp Gly Glu Ser Ile Leu Glu Leu Asn Lys Tyr Arg Ile Pro Glu 625 630 635 640	1920
AGA TTA AAA AAT AAG GAA AAA GTA AAA GTA ACC TTT ATT GGA CAT GGT Arg Leu Lys Asn Lys Glu Lys Val Lys Val Thr Phe Ile Gly His Gly 645 650 655	1968
AAA GAT GAA TTC AAC ACA AGC GAA TTT GCT AGA TTA AGT GTA GAT TCA Lys Asp Glu Phe Asn Thr Ser Glu Phe Ala Arg Leu Ser Val Asp Ser 660 665 670	2016
CTT TCC AAT GAG ATA AGT TCA TTT TTA GAT ACC ATA AAA TTA GAT ATA Leu Ser Asn Glu Ile Ser Ser Phe Leu Asp Thr Ile Lys Leu Asp Ile 675 680 685	2064
TCA CCT AAA AAT GTA GAA GTA AAC TTA CTT GGA TGT AAT ATG TTT AGT Ser Pro Lys Asn Val Glu Val Asn Leu Leu Gly Cys Asn Met Phe Ser 690 695 700	2112
TAT GAT TTT AAT GTT GAA GAA ACT TAT CCT GGG AAG TTG CTA TTA AGT Tyr Asp Phe Asn Val Glu Glu Thr Tyr Pro Gly Lys Leu Leu Leu Ser 705 710 715 720	2160
ATT ATG GAC AAA ATT ACT TCC ACT TTA CCT GAT GTA AAT AAA AAT TCT Ile Met Asp Lys Ile Thr Ser Thr Leu Pro Asp Val Asn Lys Asn Ser 725 730 735	2208



ATT ACT ATA GGA GCA AAT CAA TAT GAA GTA AGA ATT AAT AGT GAG GGA Ile Thr Ile Gly Ala Asn Gln Tyr Glu Val Arg Ile Asn Ser Glu Gly 740 745 750	2256
AGA AAA GAA CTT CTG GCT CAC TCA GGT AAA TGG ATA AAT AAA GAA GAA Arg Lys Glu Leu Leu Ala His Ser Gly Lys Trp Ile Asn Lys Glu Glu 755 760 765	2304
GCT ATT ATG AGC GAT TTA TCT AGT AAA GAA TAC ATT TTT TTT GAT TCT Ala Ile Met Ser Asp Leu Ser Ser Lys Glu Tyr Ile Phe Phe Asp Ser 770 775 780	2352
ATA GAT AAT AAG CTA AAA GCA AAG TCC AAG AAT ATT CCA GGA TTA GCA Ile Asp Asn Lys Leu Lys Ala Lys Ser Lys Asn Ile Pro Gly Leu Ala 785 790 795 800	2400
TCA ATA TCA GAA GAT ATA AAA ACA TTA TTA CTT GAT GCA AGT GTT AGT Ser Ile Ser Glu Asp Ile Lys Thr Leu Leu Leu Asp Ala Ser Val Ser 805 810 815	2448
CCT GAT ACA AAA TTT ATT TTA AAT AAT CTT AAG CTT AAT ATT GAA TCT Pro Asp Thr Lys Phe Ile Leu Asn Asn Leu Lys Leu Asn Ile Glu Ser 820 825 830	2496
TCT ATT GGG GAT TAC ATT TAT TAT GAA AAA TTA GAG CCT GTT AAA AAT Ser Ile Gly Asp Tyr Ile Tyr Tyr Glu Lys Leu Glu Pro Val Lys Asn 835 840 845	2544
ATA ATT CAC AAT TCT ATA GAT GAT TTA ATA GAT GAG TTC AAT CTA CTT Ile Ile His Asn Ser Ile Asp Asp Leu Ile Asp Glu Phe Asn Leu Leu 850 855 860	2592
GAA AAT GTA TCT GAT GAA TTA TAT GAA TTA AAA AAA TTA AAT AAT CTA Glu Asn Val Ser Asp Glu Leu Tyr Glu Leu Lys Lys Leu Asn Asn Leu 865 870 875 880	2640
GAT GAG AAG TAT TTA ATA TCT TTT GAA GAT ATC TCA AAA AAT AAT TCA Asp Glu Lys Tyr Leu Ile Ser Phe Glu Asp Ile Ser Lys Asn Asn Ser 885 890 895	2688
ACT TAC TCT GTA AGA TTT ATT AAC AAA AGT AAT GGT GAG TCA GTT TAT Thr Tyr Ser Val Arg Phe Ile Asn Lys Ser Asn Gly Glu Ser Val Tyr 900 905 910	2736
GTA GAA ACA GAA AAA GAA ATT TTT TCA AAA TAT AGC GAA CAT ATT ACA Val Glu Thr Glu Lys Glu Ile Phe Ser Lys Tyr Ser Glu His Ile Thr 915 920 925	2784
AAA GAA ATA AGT ACT ATA AAG AAT AGT ATA ATT ACA GAT GTT AAT GGT Lys Glu Ile Ser Thr Ile Lys Asn Ser Ile Ile Thr Asp Val Asn Gly 930 935 940	2832
AAT TTA TTG GAT AAT ATA CAG TTA GAT CAT ACT TCT CAA GTT AAT ACA Asn Leu Leu Asp Asn Ile Gln Leu Asp His Thr Ser Gln Val Asn Thr 945 950 955 960	2880
TTA AAC GCA GCA TTC TTT ATT CAA TCA TTA ATA GAT TAT AGT AGC AAT Leu Asn Ala Ala Phe Phe Ile Gln Ser Leu Ile Asp Tyr Ser Ser Asn 965 970 975	2928

AAA GAT GTA CTG AAT GAT TTA AGT ACC TCA GTT AAG GTT CAA CTT TAT Lys Asp Val Leu Asn Asp Leu Ser Thr Ser Val Lys Val Gln Leu Tyr 980 985 990	2976
GCT CAA CTA TTT AGT ACA GGT TTA AAT ACT ATA TAT GAC TCT ATC CAA Ala Gln Leu Phe Ser Thr Gly Leu Asn Thr Ile Tyr Asp Ser Ile Gln 995 1000 1005	3024
TTA GTA AAT TTA ATA TCA AAT GCA GTA AAT GAT ACT ATA AAT GTA CTA Leu Val Asn Leu Ile Ser Asn Ala Val Asn Asp Thr Ile Asn Val Leu 1010 1015 1020	3072
CCT ACA ATA ACA GAG GGG ATA CCT ATT GTA TCT ACT ATA TTA GAC GGA Pro Thr Ile Thr Glu Gly Ile Pro Ile Val Ser Thr Ile Leu Asp Gly 1025 1030 1035 1040	3120
ATA AAC TTA GGT GCA GCA ATT AAG GAA TTA CTA GAC GAA CAT GAC CCA Ile Asn Leu Gly Ala Ala Ile Lys Glu Leu Leu Asp Glu His Asp Pro 1045 1050 1055	3168
TTA CTA AAA AAA GAA TTA GAA GCT AAG GTG GGT GTT TTA GCA ATA AAT Leu Leu Lys Lys Glu Leu Glu Ala Lys Val Gly Val Leu Ala Ile Asn 1060 1065 1070	3216
ATG TCA TTA TCT ATA GCT GCA ACT GTA GCT TCA ATT GTT GGA ATA GGT Met Ser Leu Ser Ile Ala Ala Thr Val Ala Ser Ile Val Gly Ile Gly 1075 1080 1085	3264
GCT GAA GTT ACT ATT TTC TTA TTA CCT ATA GCT GGT ATA TCT GCA GGA Ala Glu Val Thr Ile Phe Leu Leu Pro Ile Ala Gly Ile Ser Ala Gly 1090 1095 1100	3312
ATA CCT TCA TTA GTT AAT AAT GAA TTA ATA TTG CAT GAT AAG GCA ACT Ile Pro Ser Leu Val Asn Asn Glu Leu Ile Leu His Asp Lys Ala Thr 1105 1110 1115 1120	3360
TCA GTG GTA AAC TAT TTT AAT CAT TTG TCT GAA TCT AAA AAA TAT GGC Ser Val Val Asn Tyr Phe Asn His Leu Ser Glu Ser Lys Lys Tyr Gly 1125 1130 1135	3408
CCT CTT AAA ACA GAA GAT GAT AAA ATT TTA GTT CCT ATT GAT GAT TTA Pro Leu Lys Thr Glu Asp Asp Lys Ile Leu Val Pro Ile Asp Asp Leu 1140 1145 1150	3456
GTA ATA TCA GAA ATA GAT TTT AAT AAT AAT TCG ATA AAA CTA GGA ACA Val Ile Ser Glu Ile Asp Phe Asn Asn Asn Ser Ile Lys Leu Gly Thr 1155 1160 1165	3504
TGT AAT ATA TTA GCA ATG GAG GGG GGA TCA GGA CAC ACA GTG ACT GGT Cys Asn Ile Leu Ala Met Glu Gly Gly Ser Gly His Thr Val Thr Gly 1170 1175 1180	3552
AAT ATA GAT CAC TTT TTC TCA TCT CCA TCT ATA AGT TCT CAT ATT CCT Asn Ile Asp His Phe Phe Ser Ser Pro Ser Ile Ser Ser His Ile Pro 1185 1190 1195 1200	3600
TCA TTA TCA ATT TAT TCT GCA ATA GGT ATA GAA ACA GAA AAT CTA GAT Ser Leu Ser Ile Tyr Ser Ala Ile Gly Ile Glu Thr Glu Asn Leu Asp 1205 1210 1215	3648

TTT TCA AAA AAA ATA ATG ATG TTA CCT AAT GCT CCT TCA AGA GTG TTT Phe Ser Lys Lys Ile Met Met Leu Pro Asn Ala Pro Ser Arg Val Phe 1220 1225 1230	3696
TGG TGG GAA ACT GGA GCA GTT CCA GGT TTA AGA TCA TTG GAA AAT GAC Trp Trp Glu Thr Gly Ala Val Pro Gly Leu Arg Ser Leu Glu Asn Asp 1235 1240 1245	3744
GGA ACT AGA TTA CTT GAT TCA ATA AGA GAT TTA TAC CCA GGT AAA TTT Gly Thr Arg Leu Leu Asp Ser Ile Arg Asp Leu Tyr Pro Gly Lys Phe 1250 1255 1260	3792
TAC TGG AGA TTC TAT GCT TTT TTC GAT TAT GCA ATA ACT ACA TTA AAA Tyr Trp Arg Phe Tyr Ala Phe Phe Asp Tyr Ala Ile Thr Thr Leu Lys 1265 1270 1275 1280	3840
CCA GTT TAT GAA GAC ACT AAT ATT AAA ATT AAA CTA GAT AAA GAT ACT Pro Val Tyr Glu Asp Thr Asn Ile Lys Ile Lys Leu Asp Lys Asp Thr 1285 1290 1295	3888
AGA AAC TTC ATA ATG CCA ACT ATA ACT ACT AAC GAA ATT AGA AAC AAA Arg Asn Phe Ile Met Pro Thr Ile Thr Thr Asn Glu Ile Arg Asn Lys 1300 1305 1310	3936
TTA TCT TAT TCA TTT GAT GGA GCA GGA GGA ACT TAC TCT TTA TTA TTA Leu Ser Tyr Ser Phe Asp Gly Ala Gly Gly Thr Tyr Ser Leu Leu Leu 1315 1320 1325	3984
TCT TCA TAT CCA ATA TCA ACG AAT ATA AAT TTA TCT AAA GAT GAT TTA Ser Ser Tyr Pro Ile Ser Thr Asn Ile Asn Leu Ser Lys Asp Asp Leu 1330 1335 1340	4032
TGG ATA TTT AAT ATT GAT AAT GAA GTA AGA GAA ATA TCT ATA GAA AAT Trp Ile Phe Asn Ile Asp Asn Glu Val Arg Glu Ile Ser Ile Glu Asn 1345 1350 1355 1360	4080
GGT ACT ATT AAA AAA GGA AAG TTA ATA AAA GAT GTT TTA AGT AAA ATT Gly Thr Ile Lys Lys Gly Lys Leu Ile Lys Asp Val Leu Ser Lys Ile 1365 1370 1375	4128
GAT ATA AAT AAA AAT AAA CTT ATT ATA GGC AAT CAA ACA ATA GAT TTT Asp Ile Asn Lys Asn Lys Leu Ile Ile Gly Asn Gln Thr Ile Asp Phe 1380 1385 1390	4176
TCA GGC GAT ATA GAT AAT AAA GAT AGA TAT ATA TTC TTG ACT TGT GAG Ser Gly Asp Ile Asp Asn Lys Asp Arg Tyr Ile Phe Leu Thr Cys Glu 1395 1400 1405	4224
TTA GAT GAT AAA ATT AGT TTA ATA ATA GAA ATA AAT CTT GTT GCA AAA Leu Asp Asp Lys Ile Ser Leu Ile Ile Glu Ile Asn Leu Val Ala Lys 1410 1415 1420	4272
TCT TAT AGT TTG TTA TTG TCT GGG GAT AAA AAT TAT TTG ATA TCC AAT Ser Tyr Ser Leu Leu Leu Ser Gly Asp Lys Asn Tyr Leu Ile Ser Asn 1425 1430 1435 1440	4320
TTA TCT AAT ACT ATT GAG AAA ATC AAT ACT TTA GGC CTA GAT AGT AAA Leu Ser Asn Thr Ile Glu Lys Ile Asn Thr Leu Gly Leu Asp Ser Lys 1445 1450 1455	4368

AAT ATA GCG TAC AAT TAC ACT GAT GAA TCT AAT AAT AAA TAT TTT GGA Asn Ile Ala Tyr Asn Tyr Thr Asp Glu Ser Asn Asn Lys Tyr Phe Gly 1460 1465 1470	4416
GCT ATA TCT AAA ACA AGT CAA AAA AGC ATA ATA CAT TAT AAA AAA GAC Ala Ile Ser Lys Thr Ser Gln Lys Ser Ile Ile His Tyr Lys Lys Asp 1475 1480 1485	4464
AGT AAA AAT ATA TTA GAA TTT TAT AAT GAC AGT ACA TTA GAA TTT AAC Ser Lys Asn Ile Leu Glu Phe Tyr Asn Asp Ser Thr Leu Glu Phe Asn 1490 1495 1500	4512
AGT AAA GAT TTT ATT GCT GAA GAT ATA AAT GTA TTT ATG AAA GAT GAT Ser Lys Asp Phe Ile Ala Glu Asp Ile Asn Val Phe Met Lys Asp Asp 1505 1510 1515 1520	4560
ATT AAT ACT ATA ACA GGA AAA TAC TAT GTT GAT AAT AAT ACT GAT AAA Ile Asn Thr Ile Thr Gly Lys Tyr Tyr Val Asp Asn Asn Thr Asp Lys 1525 1530 1535	4608
AGT ATA GAT TTC TCT ATT TCT TTA GTT AGT AAA AAT CAA GTA AAA GTA Ser Ile Asp Phe Ser Ile Ser Leu Val Ser Lys Asn Gln Val Lys Val 1540 1545 1550	4656
AAT GGA TTA TAT TTA AAT GAA TCC GTA TAC TCA TCT TAC CTT GAT TTT Asn Gly Leu Tyr Leu Asn Glu Ser Val Tyr Ser Ser Tyr Leu Asp Phe 1555 1560 1565	4704
GTG AAA AAT TCA GAT GGA CAC CAT AAT ACT TCT AAT TTT ATG AAT TTA Val Lys Asn Ser Asp Gly His His Asn Thr Ser Asn Phe Met Asn Leu 1570 1575 1580	4752
TTT TTG GAC AAT ATA AGT TTC TGG AAA TTG TTT GGG TTT GAA AAT ATA Phe Leu Asp Asn Ile Ser Phe Trp Lys Leu Phe Gly Phe Glu Asn Ile 1585 1590 1595 1600	4800
AAT TTT GTA ATC GAT AAA TAC TTT ACC CTT GTT GGT AAA ACT AAT CTT Asn Phe Val Ile Asp Lys Tyr Phe Thr Leu Val Gly Lys Thr Asn Leu 1605 1610 1615	4848
GGA TAT GTA GAA TTT ATT TGT GAC AAT AAT AAA AAT ATA GAT ATA TAT Gly Tyr Val Glu Phe Ile Cys Asp Asn Asn Lys Asn Ile Asp Ile Tyr 1620 1625 1630	4896
TTT GGT GAA TGG AAA ACA TCG TCA TCT AAA AGC ACT ATA TTT AGC GGA Phe Gly Glu Trp Lys Thr Ser Ser Ser Lys Ser Thr Ile Phe Ser Gly 1635 1640 1645	4944
AAT GGT AGA AAT GTT GTA GTA GAG CCT ATA TAT AAT CCT GAT ACG GGT Asn Gly Arg Asn Val Val Val Glu Pro Ile Tyr Asn Pro Asp Thr Gly 1650 1655 1660	4992
GAA GAT ATA TCT ACT TCA CTA GAT TTT TCC TAT GAA CCT CTC TAT GGA Glu Asp Ile Ser Thr Ser Leu Asp Phe Ser Tyr Glu Pro Leu Tyr Gly 1665 1670 1675 1680	5040
ATA GAT AGA TAT ATA AAT AAA GTA TTG ATA GCA CCT GAT TTA TAT ACA Ile Asp Arg Tyr Ile Asn Lys Val Leu Ile Ala Pro Asp Leu Tyr Thr 1685 1690 1695	5088

AGT TTA ATA AAT ATT AAT ACC AAT TAT TAT TCA AAT GAG TAC TAC CCT Ser Leu Ile Asn Ile Asn Thr Asn Tyr Tyr Ser Asn Glu Tyr Tyr Pro 1700 1705 1710	5136
GAG ATT ATA GTT CTT AAC CCA AAT ACA TTC CAC AAA AAA GTA AAT ATA Glu Ile Ile Val Leu Asn Pro Asn Thr Phe His Lys Lys Val Asn Ile 1715 1720 1725	5184
AAT TTA GAT AGT TCT TCT TTT GAG TAT AAA TGG TCT ACA GAA GGA AGT Asn Leu Asp Ser Ser Ser Phe Glu Tyr Lys Trp Ser Thr Glu Gly Ser 1730 1735 1740	5232
GAC TTT ATT TTA GTT AGA TAC TTA GAA GAA AGT AAT AAA AAA ATA TTA Asp Phe Ile Leu Val Arg Tyr Leu Glu Glu Ser Asn Lys Lys Ile Leu 1745 1750 1755 1760	5280
CAA AAA ATA AGA ATC AAA GGT ATC TTA TCT AAT ACT CAA TCA TTT AAT Gln Lys Ile Arg Ile Lys Gly Ile Leu Ser Asn Thr Gln Ser Phe Asn 1765 1770 1775	5328
AAA ATG AGT ATA GAT TTT AAA GAT ATT AAA AAA CTA TCA TTA GGA TAT Lys Met Ser Ile Asp Phe Lys Asp Ile Lys Lys Leu Ser Leu Gly Tyr 1780 1785 1790	5376
ATA ATG AGT AAT TTT AAA TCA TTT AAT TCT GAA AAT GAA TTA GAT AGA Ile Met Ser Asn Phe Lys Ser Phe Asn Ser Glu Asn Glu Leu Asp Arg 1795 1800 1805	5424
GAT CAT TTA GGA TTT AAA ATA ATA GAT AAT AAA ACT TAT TAC TAT GAT Asp His Leu Gly Phe Lys Ile Ile Asp Asn Lys Thr Tyr Tyr Tyr Asp 1810 1815 1820	5472
GAA GAT AGT AAA TTA GTT AAA GGA TTA ATC AAT ATA AAT AAT TCA TTA Glu Asp Ser Lys Leu Val Lys Gly Leu Ile Asn Ile Asn Asn Ser Leu 1825 1830 1835 1840	5520
TTC TAT TTT GAT CCT ATA GAA TTT AAC TTA GTA ACT GGA TGG CAA ACT Phe Tyr Phe Asp Pro Ile Glu Phe Asn Leu Val Thr Gly Trp Gln Thr 1845 1850 1855	5568
ATC AAT GGT AAA AAA TAT TAT TTT GAT ATA AAT ACT GGA GCA GCT TTA Ile Asn Gly Lys Lys Tyr Tyr Phe Asp Ile Asn Thr Gly Ala Ala Leu 1860 1865 1870	5616
ACT AGT TAT AAA ATT ATT AAT GGT AAA CAC TTT TAT TTT AAT AAT GAT Thr Ser Tyr Lys Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Asn Asp 1875 1880 1885	5664
GGT GTG ATG CAG TTG GGA GTA TTT AAA GGA CCT GAT GGA TTT GAA TAT Gly Val Met Gln Leu Gly Val Phe Lys Gly Pro Asp Gly Phe Glu Tyr 1890 1895 1900	5712
TTT GCA CCT GCC AAT ACT CAA AAT AAT AAC ATA GAA GGT CAG GCT ATA Phe Ala Pro Ala Asn Thr Gln Asn Asn Asn Ile Glu Gly Gln Ala Ile 1905 1910 1915 1920	5760
GTT TAT CAA AGT AAA TTC TTA ACT TTG AAT GGC AAA AAA TAT TAT TTT Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe 1925 1930 1935	5808

GAT AAT AAC TCA AAA GCA GTC ACT GGA TGG AGA ATT ATT AAC AAT GAG Asp Asn Asn Ser Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Glu 1940 1945 1950	5856
AAA TAT TAC TTT AAT CCT AAT AAT GCT ATT GCT GCA GTC GGA TTG CAA Lys Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala Val Gly Leu Gln 1955 1960 1965	5904
GTA ATT GAC AAT AAT AAG TAT TAT TTC AAT CCT GAC ACT GCT ATC ATC Val Ile Asp Asn Asn Lys Tyr Tyr Phe Asn Pro Asp Thr Ala Ile Ile 1970 1975 1980	5952
TCA AAA GGT TGG CAG ACT GTT AAT GGT AGT AGA TAC TAC TTT GAT ACT Ser Lys Gly Trp Gln Thr Val Asn Gly Ser Arg Tyr Tyr Phe Asp Thr 1985 1990 1995 2000	6000
GAT ACC GCT ATT GCC TTT AAT GGT TAT AAA ACT ATT GAT GGT AAA CAC Asp Thr Ala Ile Ala Phe Asn Gly Tyr Lys Thr Ile Asp Gly Lys His 2005 2010 2015	6048
TTT TAT TTT GAT AGT GAT TGT GTA GTG AAA ATA GGT GTG TTT AGT ACC Phe Tyr Phe Asp Ser Asp Cys Val Val Lys Ile Gly Val Phe Ser Thr 2020 2025 2030	6096
TCT AAT GGA TTT GAA TAT TTT GCA CCT GCT AAT ACT TAT AAT AAT AAC Ser Asn Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Tyr Asn Asn Asn 2035 2040 2045	6144
ATA GAA GGT CAG GCT ATA GTT TAT CAA AGT AAA TTC TTA ACT TTG AAT Ile Glu Gly Gln Ala Ile Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn 2050 2055 2060	6192
GGT AAA AAA TAT TAC TTT GAT AAT AAC TCA AAA GCA GTT ACC GGA TTG Gly Lys Lys Tyr Tyr Phe Asp Asn Asn Ser Lys Ala Val Thr Gly Leu 2065 2070 2075 2080	6240
CAA ACT ATT GAT AGT AAA AAA TAT TAC TTT AAT ACT AAC ACT GCT GAA Gln Thr Ile Asp Ser Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Glu 2085 2090 2095	6288
GCA GCT ACT GGA TGG CAA ACT ATT GAT GGT AAA AAA TAT TAC TTT AAT Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn 2100 2105 2110	6336
ACT AAC ACT GCT GAA GCA GCT ACT GGA TGG CAA ACT ATT GAT GGT AAA Thr Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys 2115 2120 2125	6384
AAA TAT TAC TTT AAT ACT AAC ACT GCT ATA GCT TCA ACT GGT TAT ACA Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Ile Ala Ser Thr Gly Tyr Thr 2130 2135 2140	6432
ATT ATT AAT GGT AAA CAT TTT TAT TTT AAT ACT GAT GGT ATT ATG CAG Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Thr Asp Gly Ile Met Gln 2145 2150 2155 2160	6480
ATA GGA GTG TTT AAA GGA CCT AAT GGA TTT GAA TAT TTT GCA CCT GCT Ile Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala Pro Ala 2165 2170 2175	6528

AAT ACG GAT GCT AAC AAC ATA GAA GGT CAA GCT ATA CTT TAC CAA AAT Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile Leu Tyr Gln Asn 2180 2185 2190	6576
GAA TTC TTA ACT TTG AAT GGT AAA AAA TAT TAC TTT GGT AGT GAC TCA Glu Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Gly Ser Asp Ser 2195 2200 2205	6624
AAA GCA GTT ACT GGA TGG AGA ATT ATT AAC AAT AAG AAA TAT TAC TTT Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Lys Lys Tyr Tyr Phe 2210 2215 2220	6672
AAT CCT AAT AAT GCT ATT GCT GCA ATT CAT CTA TGC ACT ATA AAT AAT Asn Pro Asn Asn Ala Ile Ala Ala Ile His Leu Cys Thr Ile Asn Asn 2225 2230 2235 2240	6720
GAC AAG TAT TAC TTT AGT TAT GAT GGA ATT CTT CAA AAT GGA TAT ATT Asp Lys Tyr Tyr Phe Ser Tyr Asp Gly Ile Leu Gln Asn Gly Tyr Ile 2245 2250 2255	6768
ACT ATT GAA AGA AAT AAT TTC TAT TTT GAT GCT AAT AAT GAA TCT AAA Thr Ile Glu Arg Asn Asn Phe Tyr Phe Asp Ala Asn Asn Glu Ser Lys 2260 2265 2270	6816
ATG GTA ACA GGA GTA TTT AAA GGA CCT AAT GGA TTT GAG TAT TTT GCA Met Val Thr Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala 2275 2280 2285	6864
CCT GCT AAT ACT CAC AAT AAT AAC ATA GAA GGT CAG GCT ATA GTT TAC Pro Ala Asn Thr His Asn Asn Asn Ile Glu Gly Gln Ala Ile Val Tyr 2290 2295 2300	6912
CAG AAC AAA TTC TTA ACT TTG AAT GGC AAA AAA TAT TAT TTT GAT AAT Gln Asn Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp Asn 2305 2310 2315 2320	6960
GAC TCA AAA GCA GTT ACT GGA TGG CAA ACC ATT GAT GGT AAA AAA TAT Asp Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr 2325 2330 2335	7008
TAC TTT AAT CTT AAC ACT GCT GAA GCA GCT ACT GGA TGG CAA ACT ATT Tyr Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile 2340 2345 2350	7056
GAT GGT AAA AAA TAT TAC TTT AAT CTT AAC ACT GCT GAA GCA GCT ACT Asp Gly Lys Lys Tyr Tyr Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr 2355 2360 2365	7104
GGA TGG CAA ACT ATT GAT GGT AAA AAA TAT TAC TTT AAT ACT AAC ACT Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr 2370 2375 2380	7152
TTC ATA GCC TCA ACT GGT TAT ACA AGT ATT AAT GGT AAA CAT TTT TAT Phe Ile Ala Ser Thr Gly Tyr Thr Ser Ile Asn Gly Lys His Phe Tyr 2385 2390 2395 2400	7200
TTT AAT ACT GAT GGT ATT ATG CAG ATA GGA GTG TTT AAA GGA CCT AAT Phe Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro Asn 2405 2410 2415	7248

GGA TTT GAA TAC TTT GCA CCT GCT AAT ACG GAT GCT AAC AAC ATA GAA Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu 2420 2425 2430	7296
GGT CAA GCT ATA CTT TAC CAA AAT AAA TTC TTA ACT TTG AAT GGT AAA Gly Gln Ala Ile Leu Tyr Gln Asn Lys Phe Leu Thr Leu Asn Gly Lys 2435 2440 2445	7344
AAA TAT TAC TTT GGT AGT GAC TCA AAA GCA GTT ACC GGA CTG CGA ACT Lys Tyr Tyr Phe Gly Ser Asp Ser Lys Ala Val Thr Gly Leu Arg Thr 2450 2455 2460	7392
ATT GAT GGT AAA AAA TAT TAC TTT AAT ACT AAC ACT GCT GTT GCA GTT Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Val Ala Val 2465 2470 2475 2480	7440
ACT GGA TGG CAA ACT ATT AAT GGT AAA AAA TAC TAC TTT AAT ACT AAC Thr Gly Trp Gln Thr Ile Asn Gly Lys Lys Tyr Tyr Phe Asn Thr Asn 2485 2490 2495	7488
ACT TCT ATA GCT TCA ACT GGT TAT ACA ATT ATT AGT GGT AAA CAT TTT Thr Ser Ile Ala Ser Thr Gly Tyr Thr Ile Ile Ser Gly Lys His Phe 2500 2505 2510	7536
TAT TTT AAT ACT GAT GGT ATT ATG CAG ATA GGA GTG TTT AAA GGA CCT Tyr Phe Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro 2515 2520 2525	7584
GAT GGA TTT GAA TAC TTT GCA CCT GCT AAT ACA GAT GCT AAC AAT ATA Asp Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile 2530 2535 2540	7632
GAA GGT CAA GCT ATA CGT TAT CAA AAT AGA TTC CTA TAT TTA CAT GAC Glu Gly Gln Ala Ile Arg Tyr Gln Asn Arg Phe Leu Tyr Leu His Asp 2545 2550 2555 2560	7680
AAT ATA TAT TAT TTT GGT AAT AAT TCA AAA GCG GCT ACT GGT TGG GTA Asn Ile Tyr Tyr Phe Gly Asn Asn Ser Lys Ala Ala Thr Gly Trp Val 2565 2570 2575	7728
ACT ATT GAT GGT AAT AGA TAT TAC TTC GAG CCT AAT ACA GCT ATG GGT Thr Ile Asp Gly Asn Arg Tyr Tyr Phe Glu Pro Asn Thr Ala Met Gly 2580 2585 2590	7776
GCG AAT GGT TAT AAA ACT ATT GAT AAT AAA AAT TTT TAC TTT AGA AAT Ala Asn Gly Tyr Lys Thr Ile Asp Asn Lys Asn Phe Tyr Phe Arg Asn 2595 2600 2605	7824
GGT TTA CCT CAG ATA GGA GTG TTT AAA GGG TCT AAT GGA TTT GAA TAC Gly Leu Pro Gln Ile Gly Val Phe Lys Gly Ser Asn Gly Phe Glu Tyr 2610 2615 2620	7872
TTT GCA CCT GCT AAT ACG GAT GCT AAC AAT ATA GAA GGT CAA GCT ATA Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile 2625 2630 2635 2640	7920
CGT TAT CAA AAT AGA TTC CTA CAT TTA CTT GGA AAA ATA TAT TAC TTT Arg Tyr Gln Asn Arg Phe Leu His Leu Leu Gly Lys Ile Tyr Tyr Phe 2645 2650 2655	7968



GGT AAT AAT TCA AAA GCA GTT ACT GGA TGG CAA ACT ATT AAT GGT AAA 8016  
Gly Asn Asn Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asn Gly Lys  
2660 2665 2670

GTA TAT TAC TTT ATG CCT GAT ACT GCT ATG GCT GCA GCT GGT GGA CTT 8064  
Val Tyr Tyr Phe Met Pro Asp Thr Ala Met Ala Ala Gly Gly Leu  
2675 2680 2685

TTC GAG ATT GAT GGT GTT ATA TAT TTC TTT GGT GTT GAT GGA GTA AAA 8112  
Phe Glu Ile Asp Gly Val Ile Tyr Phe Phe Gly Val Asp Gly Val Lys  
2690 2695 2700

GCC CCT GGG ATA TAT GGC TAA  
Ala Pro Gly Ile Tyr Gly  
2705 2710

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2710 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ser Leu Ile Ser Lys Glu Glu Leu Ile Lys Leu Ala Tyr Ser Ile  
1 5 10 15

Arg Pro Arg Glu Asn Glu Tyr Lys Thr Ile Leu Thr Asn Leu Asp Glu  
20 25 30

Tyr Asn Lys Leu Thr Thr Asn Asn Asn Glu Asn Lys Tyr Leu Gln Leu  
35 40 45

Lys Lys Leu Asn Glu Ser Ile Asp Val Phe Met Asn Lys Tyr Lys Thr  
50 55 60

Ser<sub>65</sub> Ser Arg Asn Arg Ala<sub>70</sub> Leu Ser Asn Leu Lys<sub>75</sub> Lys Asp Ile Leu Lys<sub>80</sub>

Glu Val Ile Leu Ile Lys Asn Ser Asn Thr Ser Pro Val Glu Lys Asn  
85 90 95

Leu His Phe Val Trp Ile Gly Gly Glu Val Ser Asp Ile Ala Leu Glu  
100 105 110

Tyr Ile Lys Gln Trp Ala Asp Ile Asn Ala Glu Tyr Asn Ile Lys Leu  
115 120 125

Trp Tyr Asp Ser Glu Ala Phe Leu Val Asn Thr Leu Lys Lys Ala Ile  
130 135 140

Val Glu Ser Ser Thr Thr Glu Ala Leu Gln Leu Leu Glu Glu Glu Ile  
145 150 155 160

Gln Asn Pro Gln Phe Asp Asn Met Lys Phe Tyr Lys Lys Arg Met Glu  
165 170 175

Phe Ile Tyr Asp Arg Gln Lys Arg Phe Ile Asn Tyr Tyr Lys Ser Gln  
180 185 190

Ile Asn Lys Pro Thr Val Pro Thr Ile Asp Asp Ile Ile Lys Ser His  
 195 200 205  
 Leu Val Ser Glu Tyr Asn Arg Asp Glu Thr Val Leu Glu Ser Tyr Arg  
 210 215 220  
 Thr Asn Ser Leu Arg Lys Ile Asn Ser Asn His Gly Ile Asp Ile Arg  
 225 230 235 240  
 Ala Asn Ser Leu Phe Thr Glu Gln Glu Leu Leu Asn Ile Tyr Ser Gln  
 245 250 255  
 Glu Leu Leu Asn Arg Gly Asn Leu Ala Ala Ala Ser Asp Ile Val Arg  
 260 265 270  
 Leu Leu Ala Leu Lys Asn Phe Gly Gly Val Tyr Leu Asp Val Asp Met  
 275 280 285  
 Leu Pro Gly Ile His Ser Asp Leu Phe Lys Thr Ile Ser Arg Pro Ser  
 290 295 300  
 Ser Ile Gly Leu Asp Arg Trp Glu Met Ile Lys Leu Glu Ala Ile Met  
 305 310 315 320  
 Lys Tyr Lys Lys Tyr Ile Asn Asn Tyr Thr Ser Glu Asn Phe Asp Lys  
 325 330 335  
 Leu Asp Gln Gln Leu Lys Asp Asn Phe Lys Leu Ile Ile Glu Ser Lys  
 340 345 350  
 Ser Glu Lys Ser Glu Ile Phe Ser Lys Leu Glu Asn Leu Asn Val Ser  
 355 360 365  
 Asp Leu Glu Ile Lys Ile Ala Phe Ala Leu Gly Ser Val Ile Asn Gln  
 370 375 380  
 Ala Leu Ile Ser Lys Gln Gly Ser Tyr Leu Thr Asn Leu Val Ile Glu  
 385 390 395 400  
 Gln Val Lys Asn Arg Tyr Gln Phe Leu Asn Gln His Leu Asn Pro Ala  
 405 410 415  
 Ile Glu Ser Asp Asn Asn Phe Thr Asp Thr Thr Lys Ile Phe His Asp  
 420 425 430  
 Ser Leu Phe Asn Ser Ala Thr Ala Glu Asn Ser Met Phe Leu Thr Lys  
 435 440 445  
 Ile Ala Pro Tyr Leu Gln Val Gly Phe Met Pro Glu Ala Arg Ser Thr  
 450 455 460  
 Ile Ser Leu Ser Gly Pro Gly Ala Tyr Ala Ser Ala Tyr Tyr Asp Phe  
 465 470 475 480  
 Ile Asn Leu Gln Glu Asn Thr Ile Glu Lys Thr Leu Lys Ala Ser Asp  
 485 490 495  
 Leu Ile Glu Phe Lys Phe Pro Glu Asn Asn Leu Ser Gln Leu Thr Glu  
 500 505 510  
 Gln Glu Ile Asn Ser Leu Trp Ser Phe Asp Gln Ala Ser Ala Lys Tyr  
 515 520 525

Gln Phe Glu Lys Tyr Val Arg Asp Tyr Thr Gly Gly Ser Leu Ser Glu  
 530 535 540  
 Asp Asn Gly Val Asp Phe Asn Lys Asn Thr Ala Leu Asp Lys Asn Tyr  
 545 550 555 560  
 Leu Leu Asn Asn Lys Ile Pro Ser Asn Asn Val Glu Glu Ala Gly Ser  
 565 570 575  
 Lys Asn Tyr Val His Tyr Ile Ile Gln Leu Gln Gly Asp Asp Ile Ser  
 580 585 590  
 Tyr Glu Ala Thr Cys Asn Leu Phe Ser Lys Asn Pro Lys Asn Ser Ile  
 595 600 605  
 Ile Ile Gln Arg Asn Met Asn Glu Ser Ala Lys Ser Tyr Phe Leu Ser  
 610 615 620  
 Asp Asp Gly Glu Ser Ile Leu Glu Leu Asn Lys Tyr Arg Ile Pro Glu  
 625 630 635 640  
 Arg Leu Lys Asn Lys Glu Lys Val Lys Val Thr Phe Ile Gly His Gly  
 645 650 655  
 Lys Asp Glu Phe Asn Thr Ser Glu Phe Ala Arg Leu Ser Val Asp Ser  
 660 665 670  
 Leu Ser Asn Glu Ile Ser Ser Phe Leu Asp Thr Ile Lys Leu Asp Ile  
 675 680 685  
 Ser Pro Lys Asn Val Glu Val Asn Leu Leu Gly Cys Asn Met Phe Ser  
 690 695 700  
 Tyr Asp Phe Asn Val Glu Glu Thr Tyr Pro Gly Lys Leu Leu Leu Ser  
 705 710 715 720  
 Ile Met Asp Lys Ile Thr Ser Thr Leu Pro Asp Val Asn Lys Asn Ser  
 725 730 735  
 Ile Thr Ile Gly Ala Asn Gln Tyr Glu Val Arg Ile Asn Ser Glu Gly  
 740 745 750  
 Arg Lys Glu Leu Leu Ala His Ser Gly Lys Trp Ile Asn Lys Glu Glu  
 755 760 765  
 Ala Ile Met Ser Asp Leu Ser Ser Lys Glu Tyr Ile Phe Phe Asp Ser  
 770 775 780  
 Ile Asp Asn Lys Leu Lys Ala Lys Ser Lys Asn Ile Pro Gly Leu Ala  
 785 790 795 800  
 Ser Ile Ser Glu Asp Ile Lys Thr Leu Leu Leu Asp Ala Ser Val Ser  
 805 810 815  
 Pro Asp Thr Lys Phe Ile Leu Asn Asn Leu Lys Leu Asn Ile Glu Ser  
 820 825 830  
 Ser Ile Gly Asp Tyr Ile Tyr Tyr Glu Lys Leu Glu Pro Val Lys Asn  
 835 840 845  
 Ile Ile His Asn Ser Ile Asp Asp Leu Ile Asp Glu Phe Asn Leu Leu  
 850 855 860

Glu Asn Val Ser Asp Glu Leu Tyr Glu Leu Lys Lys Leu Asn Asn Leu  
 865 870 875 880  
 Asp Glu Lys Tyr Leu Ile Ser Phe Glu Asp Ile Ser Lys Asn Asn Ser  
 885 890 895  
 Thr Tyr Ser Val Arg Phe Ile Asn Lys Ser Asn Gly Glu Ser Val Tyr  
 900 905 910  
 Val Glu Thr Glu Lys Glu Ile Phe Ser Lys Tyr Ser Glu His Ile Thr  
 915 920 925  
 Lys Glu Ile Ser Thr Ile Lys Asn Ser Ile Ile Thr Asp Val Asn Gly  
 930 935 940  
 Asn Leu Leu Asp Asn Ile Gln Leu Asp His Thr Ser Gln Val Asn Thr  
 945 950 955 960  
 Leu Asn Ala Ala Phe Phe Ile Gln Ser Leu Ile Asp Tyr Ser Ser Asn  
 965 970 975  
 Lys Asp Val Leu Asn Asp Leu Ser Thr Ser Val Lys Val Gln Leu Tyr  
 980 985 990  
 Ala Gln Leu Phe Ser Thr Gly Leu Asn Thr Ile Tyr Asp Ser Ile Gln  
 995 1000 1005  
 Leu Val Asn Leu Ile Ser Asn Ala Val Asn Asp Thr Ile Asn Val Leu  
 1010 1015 1020  
 Pro Thr Ile Thr Glu Gly Ile Pro Ile Val Ser Thr Ile Leu Asp Gly  
 1025 1030 1035 1040  
 Ile Asn Leu Gly Ala Ala Ile Lys Glu Leu Leu Asp Glu His Asp Pro  
 1045 1050 1055  
 Leu Leu Lys Lys Glu Leu Glu Ala Lys Val Gly Val Leu Ala Ile Asn  
 1060 1065 1070  
 Met Ser Leu Ser Ile Ala Ala Thr Val Ala Ser Ile Val Gly Ile Gly  
 1075 1080 1085  
 Ala Glu Val Thr Ile Phe Leu Leu Pro Ile Ala Gly Ile Ser Ala Gly  
 1090 1095 1100  
 Ile Pro Ser Leu Val Asn Asn Glu Leu Ile Leu His Asp Lys Ala Thr  
 1105 1110 1115 1120  
 Ser Val Val Asn Tyr Phe Asn His Leu Ser Glu Ser Lys Lys Tyr Gly  
 1125 1130 1135  
 Pro Leu Lys Thr Glu Asp Asp Lys Ile Leu Val Pro Ile Asp Asp Leu  
 1140 1145 1150  
 Val Ile Ser Glu Ile Asp Phe Asn Asn Asn Ser Ile Lys Leu Gly Thr  
 1155 1160 1165  
 Cys Asn Ile Leu Ala Met Glu Gly Gly Ser Gly His Thr Val Thr Gly  
 1170 1175 1180  
 Asn Ile Asp His Phe Phe Ser Ser Pro Ser Ile Ser Ser His Ile Pro  
 1185 1190 1195 1200

Ser Leu Ser Ile Tyr Ser Ala Ile Gly Ile Glu Thr Glu Asn Leu Asp  
 1205 1210 1215  
 Phe Ser Lys Lys Ile Met Met Leu Pro Asn Ala Pro Ser Arg Val Phe  
 1220 1225 1230  
 Trp Trp Glu Thr Gly Ala Val Pro Gly Leu Arg Ser Leu Glu Asn Asp  
 1235 1240 1245  
 Gly Thr Arg Leu Leu Asp Ser Ile Arg Asp Leu Tyr Pro Gly Lys Phe  
 1250 1255 1260  
 Tyr Trp Arg Phe Tyr Ala Phe Phe Asp Tyr Ala Ile Thr Thr Leu Lys  
 1265 1270 1275 1280  
 Pro Val Tyr Glu Asp Thr Asn Ile Lys Ile Lys Leu Asp Lys Asp Thr  
 1285 1290 1295  
 Arg Asn Phe Ile Met Pro Thr Ile Thr Thr Asn Glu Ile Arg Asn Lys  
 1300 1305 1310  
 Leu Ser Tyr Ser Phe Asp Gly Ala Gly Gly Thr Tyr Ser Leu Leu Leu  
 1315 1320 1325  
 Ser Ser Tyr Pro Ile Ser Thr Asn Ile Asn Leu Ser Lys Asp Asp Leu  
 1330 1335 1340  
 Trp Ile Phe Asn Ile Asp Asn Glu Val Arg Glu Ile Ser Ile Glu Asn  
 1345 1350 1355 1360  
 Gly Thr Ile Lys Lys Gly Lys Leu Ile Lys Asp Val Leu Ser Lys Ile  
 1365 1370 1375  
 Asp Ile Asn Lys Asn Lys Leu Ile Ile Gly Asn Gln Thr Ile Asp Phe  
 1380 1385 1390  
 Ser Gly Asp Ile Asp Asn Lys Asp Arg Tyr Ile Phe Leu Thr Cys Glu  
 1395 1400 1405  
 Leu Asp Asp Lys Ile Ser Leu Ile Ile Glu Ile Asn Leu Val Ala Lys  
 1410 1415 1420  
 Ser Tyr Ser Leu Leu Leu Ser Gly Asp Lys Asn Tyr Leu Ile Ser Asn  
 1425 1430 1435 1440  
 Leu Ser Asn Thr Ile Glu Lys Ile Asn Thr Leu Gly Leu Asp Ser Lys  
 1445 1450 1455  
 Asn Ile Ala Tyr Asn Tyr Thr Asp Glu Ser Asn Asn Lys Tyr Phe Gly  
 1460 1465 1470  
 Ala Ile Ser Lys Thr Ser Gln Lys Ser Ile Ile His Tyr Lys Lys Asp  
 1475 1480 1485  
 Ser Lys Asn Ile Leu Glu Phe Tyr Asn Asp Ser Thr Leu Glu Phe Asn  
 1490 1495 1500  
 Ser Lys Asp Phe Ile Ala Glu Asp Ile Asn Val Phe Met Lys Asp Asp  
 1505 1510 1515 1520  
 Ile Asn Thr Ile Thr Gly Lys Tyr Tyr Val Asp Asn Asn Thr Asp Lys  
 1525 1530 1535

Ser Ile Asp Phe Ser Ile Ser Leu Val Ser Lys Asn Gln Val Lys Val  
 1540 1545 1550  
 Asn Gly Leu Tyr Leu Asn Glu Ser Val Tyr Ser Ser Tyr Leu Asp Phe  
 1555 1560 1565  
 Val Lys Asn Ser Asp Gly His His Asn Thr Ser Asn Phe Met Asn Leu  
 1570 1575 1580  
 Phe Leu Asp Asn Ile Ser Phe Trp Lys Leu Phe Gly Phe Glu Asn Ile  
 1585 1590 1595 1600  
 Asn Phe Val Ile Asp Lys Tyr Phe Thr Leu Val Gly Lys Thr Asn Leu  
 1605 1610 1615  
 Gly Tyr Val Glu Phe Ile Cys Asp Asn Asn Lys Asn Ile Asp Ile Tyr  
 1620 1625 1630  
 Phe Gly Glu Trp Lys Thr Ser Ser Ser Lys Ser Thr Ile Phe Ser Gly  
 1635 1640 1645  
 Asn Gly Arg Asn Val Val Val Glu Pro Ile Tyr Asn Pro Asp Thr Gly  
 1650 1655 1660  
 Glu Asp Ile Ser Thr Ser Leu Asp Phe Ser Tyr Glu Pro Leu Tyr Gly  
 1665 1670 1675 1680  
 Ile Asp Arg Tyr Ile Asn Lys Val Leu Ile Ala Pro Asp Leu Tyr Thr  
 1685 1690 1695  
 Ser Leu Ile Asn Ile Asn Thr Asn Tyr Tyr Ser Asn Glu Tyr Tyr Pro  
 1700 1705 1710  
 Glu Ile Ile Val Leu Asn Pro Asn Thr Phe His Lys Lys Val Asn Ile  
 1715 1720 1725  
 Asn Leu Asp Ser Ser Ser Phe Glu Tyr Lys Trp Ser Thr Glu Gly Ser  
 1730 1735 1740  
 Asp Phe Ile Leu Val Arg Tyr Leu Glu Glu Ser Asn Lys Lys Ile Leu  
 1745 1750 1755 1760  
 Gln Lys Ile Arg Ile Lys Gly Ile Leu Ser Asn Thr Gln Ser Phe Asn  
 1765 1770 1775  
 Lys Met Ser Ile Asp Phe Lys Asp Ile Lys Lys Leu Ser Leu Gly Tyr  
 1780 1785 1790  
 Ile Met Ser Asn Phe Lys Ser Phe Asn Ser Glu Asn Glu Leu Asp Arg  
 1795 1800 1805  
 Asp His Leu Gly Phe Lys Ile Ile Asp Asn Lys Thr Tyr Tyr Tyr Asp  
 1810 1815 1820  
 Glu Asp Ser Lys Leu Val Lys Gly Leu Ile Asn Ile Asn Asn Ser Leu  
 1825 1830 1835 1840  
 Phe Tyr Phe Asp Pro Ile Glu Phe Asn Leu Val Thr Gly Trp Gln Thr  
 1845 1850 1855  
 Ile Asn Gly Lys Lys Tyr Tyr Phe Asp Ile Asn Thr Gly Ala Ala Leu  
 1860 1865 1870

Thr Ser Tyr Lys Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Asn Asp  
 1875 1880 1885  
 Gly Val Met Gln Leu Gly Val Phe Lys Gly Pro Asp Gly Phe Glu Tyr  
 1890 1895 1900  
 Phe Ala Pro Ala Asn Thr Gln Asn Asn Asn Ile Glu Gly Gln Ala Ile  
 1905 1910 1915 1920  
 Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe  
 1925 1930 1935  
 Asp Asn Asn Ser Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Glu  
 1940 1945 1950  
 Lys Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala Val Gly Leu Gln  
 1955 1960 1965  
 Val Ile Asp Asn Asn Lys Tyr Tyr Phe Asn Pro Asp Thr Ala Ile Ile  
 1970 1975 1980  
 Ser Lys Gly Trp Gln Thr Val Asn Gly Ser Arg Tyr Tyr Phe Asp Thr  
 1985 1990 1995 2000  
 Asp Thr Ala Ile Ala Phe Asn Gly Tyr Lys Thr Ile Asp Gly Lys His  
 2005 2010 2015  
 Phe Tyr Phe Asp Ser Asp Cys Val Val Lys Ile Gly Val Phe Ser Thr  
 2020 2025 2030  
 Ser Asn Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Tyr Asn Asn Asn  
 2035 2040 2045  
 Ile Glu Gly Gln Ala Ile Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn  
 2050 2055 2060  
 Gly Lys Lys Tyr Tyr Phe Asp Asn Asn Ser Lys Ala Val Thr Gly Leu  
 2065 2070 2075 2080  
 Gln Thr Ile Asp Ser Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Glu  
 2085 2090 2095  
 Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn  
 2100 2105 2110  
 Thr Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys  
 2115 2120 2125  
 Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Ile Ala Ser Thr Gly Tyr Thr  
 2130 2135 2140  
 Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Thr Asp Gly Ile Met Gln  
 2145 2150 2155 2160  
 Ile Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala Pro Ala  
 2165 2170 2175  
 Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile Leu Tyr Gln Asn  
 2180 2185 2190  
 Glu Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Gly Ser Asp Ser  
 2195 2200 2205

Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Lys Lys Tyr Tyr Phe  
 2210 2215 2220  
 Asn Pro Asn Asn Ala Ile Ala Ala Ile His Leu Cys Thr Ile Asn Asn  
 2225 2230 2235 2240  
 Asp Lys Tyr Tyr Phe Ser Tyr Asp Gly Ile Leu Gln Asn Gly Tyr Ile  
 2245 2250 2255  
 Thr Ile Glu Arg Asn Asn Phe Tyr Phe Asp Ala Asn Asn Glu Ser Lys  
 2260 2265 2270  
 Met Val Thr Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala  
 2275 2280 2285  
 Pro Ala Asn Thr His Asn Asn Asn Ile Glu Gly Gln Ala Ile Val Tyr  
 2290 2295 2300  
 Gln Asn Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp Asn  
 2305 2310 2315 2320  
 Asp Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr  
 2325 2330 2335  
 Tyr Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile  
 2340 2345 2350  
 Asp Gly Lys Lys Tyr Tyr Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr  
 2355 2360 2365  
 Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr  
 2370 2375 2380  
 Phe Ile Ala Ser Thr Gly Tyr Thr Ser Ile Asn Gly Lys His Phe Tyr  
 2385 2390 2395 2400  
 Phe Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro Asn  
 2405 2410 2415  
 Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu  
 2420 2425 2430  
 Gly Gln Ala Ile Leu Tyr Gln Asn Lys Phe Leu Thr Leu Asn Gly Lys  
 2435 2440 2445  
 Lys Tyr Tyr Phe Gly Ser Asp Ser Lys Ala Val Thr Gly Leu Arg Thr  
 2450 2455 2460  
 Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Val Ala Val  
 2465 2470 2475 2480  
 Thr Gly Trp Gln Thr Ile Asn Gly Lys Lys Tyr Tyr Phe Asn Thr Asn  
 2485 2490 2495  
 Thr Ser Ile Ala Ser Thr Gly Tyr Thr Ile Ile Ser Gly Lys His Phe  
 2500 2505 2510  
 Tyr Phe Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro  
 2515 2520 2525  
 Asp Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile  
 2530 2535 2540



Glu Gly Gln Ala Ile Arg Tyr Gln Asn Arg Phe Leu Tyr Leu His Asp  
 2545                      2550                      2555                      2560  
 Asn Ile Tyr Tyr Phe Gly Asn Asn Ser Lys Ala Ala Thr Gly Trp Val  
                          2565                      2570                      2575  
 Thr Ile Asp Gly Asn Arg Tyr Tyr Phe Glu Pro Asn Thr Ala Met Gly  
                          2580                      2585                      2590  
 Ala Asn Gly Tyr Lys Thr Ile Asp Asn Lys Asn Phe Tyr Phe Arg Asn  
                          2595                      2600                      2605  
 Gly Leu Pro Gln Ile Gly Val Phe Lys Gly Ser Asn Gly Phe Glu Tyr  
                          2610                      2615                      2620  
 Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile  
 2625                      2630                      2635                      2640  
 Arg Tyr Gln Asn Arg Phe Leu His Leu Leu Gly Lys Ile Tyr Tyr Phe  
                          2645                      2650                      2655  
 Gly Asn Asn Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asn Gly Lys  
                          2660                      2665                      2670  
 Val Tyr Tyr Phe Met Pro Asp Thr Ala Met Ala Ala Ala Gly Gly Leu  
                          2675                      2680                      2685  
 Phe Glu Ile Asp Gly Val Ile Tyr Phe Phe Gly Val Asp Gly Val Lys  
                          2690                      2695                      2700  
 Ala Pro Gly Ile Tyr Gly  
 2705                      2710

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 811 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Tyr Lys Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Asn Asp Gly  
 1                      5                      10                      15  
 Val Met Gln Leu Gly Val Phe Lys Gly Pro Asp Gly Phe Glu Tyr Phe  
                          20                      25                      30  
 Ala Pro Ala Asn Thr Gln Asn Asn Asn Ile Glu Gly Gln Ala Ile Val  
                          35                      40                      45  
 Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp  
                          50                      55                      60  
 Asn Asn Ser Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Glu Lys  
 65                      70                      75                      80  
 Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala Val Gly Leu Gln Val  
                          85                      90                      95

Ile Asp Asn Asn Lys Tyr Tyr Phe Asn Pro Asp Thr Ala Ile Ile Ser  
 100 105 110  
 Lys Gly Trp Gln Thr Val Asn Gly Ser Arg Tyr Tyr Phe Asp Thr Asp  
 115 120 125  
 Thr Ala Ile Ala Phe Asn Gly Tyr Lys Thr Ile Asp Gly Lys His Phe  
 130 135 140  
 Tyr Phe Asp Ser Asp Cys Val Val Lys Ile Gly Val Phe Ser Thr Ser  
 145 150 155 160  
 Asn Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Tyr Asn Asn Asn Ile  
 165 170 175  
 Glu Gly Gln Ala Ile Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly  
 180 185 190  
 Lys Lys Tyr Tyr Phe Asp Asn Asn Ser Lys Ala Val Thr Gly Leu Gln  
 195 200 205  
 Thr Ile Asp Ser Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Glu Ala  
 210 215 220  
 Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr  
 225 230 235 240  
 Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys  
 245 250 255  
 Tyr Tyr Phe Asn Thr Asn Thr Ala Ile Ala Ser Thr Gly Tyr Thr Ile  
 260 265 270  
 Ile Asn Gly Lys His Phe Tyr Phe Asn Thr Asp Gly Ile Met Gln Ile  
 275 280 285  
 Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala Pro Ala Asn  
 290 295 300  
 Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile Leu Tyr Gln Asn Glu  
 305 310 315 320  
 Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Gly Ser Asp Ser Lys  
 325 330 335  
 Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Lys Lys Tyr Tyr Phe Asn  
 340 345 350  
 Pro Asn Asn Ala Ile Ala Ala Ile His Leu Cys Thr Ile Asn Asn Asp  
 355 360 365  
 Lys Tyr Tyr Phe Ser Tyr Asp Gly Ile Leu Gln Asn Gly Tyr Ile Thr  
 370 375 380  
 Ile Glu Arg Asn Asn Phe Tyr Phe Asp Ala Asn Asn Glu Ser Lys Met  
 385 390 395 400  
 Val Thr Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala Pro  
 405 410 415  
 Ala Asn Thr His Asn Asn Asn Ile Glu Gly Gln Ala Ile Val Tyr Gln  
 420 425 430

Asn Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp Asn Asp  
 435 440 445  
 Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr  
 450 455 460  
 Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile Asp  
 465 470 475 480  
 Gly Lys Lys Tyr Tyr Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr Gly  
 485 490 495  
 Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Phe  
 500 505 510  
 Ile Ala Ser Thr Gly Tyr Thr Ser Ile Asn Gly Lys His Phe Tyr Phe  
 515 520 525  
 Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro Asn Gly  
 530 535 540  
 Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu Gly  
 545 550 555 560  
 Gln Ala Ile Leu Tyr Gln Asn Lys Phe Leu Thr Leu Asn Gly Lys Lys  
 565 570 575  
 Tyr Tyr Phe Gly Ser Asp Ser Lys Ala Val Thr Gly Leu Arg Thr Ile  
 580 585 590  
 Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Val Ala Val Thr  
 595 600 605  
 Gly Trp Gln Thr Ile Asn Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr  
 610 615 620  
 Ser Ile Ala Ser Thr Gly Tyr Thr Ile Ile Ser Gly Lys His Phe Tyr  
 625 630 635 640  
 Phe Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro Asp  
 645 650 655  
 Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu  
 660 665 670  
 Gly Gln Ala Ile Arg Tyr Gln Asn Arg Phe Leu Tyr Leu His Asp Asn  
 675 680 685  
 Ile Tyr Tyr Phe Gly Asn Asn Ser Lys Ala Ala Thr Gly Trp Val Thr  
 690 695 700  
 Ile Asp Gly Asn Arg Tyr Tyr Phe Glu Pro Asn Thr Ala Met Gly Ala  
 705 710 715 720  
 Asn Gly Tyr Lys Thr Ile Asp Asn Lys Asn Phe Tyr Phe Arg Asn Gly  
 725 730 735  
 Leu Pro Gln Ile Gly Val Phe Lys Gly Ser Asn Gly Phe Glu Tyr Phe  
 740 745 750  
 Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile Arg  
 755 760 765

Tyr Gln Asn Arg Phe Leu His Leu Leu Gly Lys Ile Tyr Tyr Phe Gly  
770 775 780  
Asn Asn Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asn Gly Lys Val  
785 790 795 800  
Tyr Tyr Phe Met Pro Asp Thr Ala Met Ala Ala  
805 810

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 91 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Tyr Lys Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Asn Asp Gly  
1 5 10 15  
Val Met Gln Leu Gly Val Phe Lys Gly Pro Asp Gly Phe Glu Tyr Phe  
20 25 30  
Ala Pro Ala Asn Thr Gln Asn Asn Asn Ile Glu Gly Gln Ala Ile Val  
35 40 45  
Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp  
50 55 60  
Asn Asn Ser Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Glu Lys  
65 70 75 80  
Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala  
85 90

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7101 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 1..7098

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATG AGT TTA GTT AAT AGA AAA CAG TTA GAA AAA ATG GCA AAT GTA AGA 48  
Met Ser Leu Val Asn Arg Lys Gln Leu Glu Lys Met Ala Asn Val Arg  
1 5 10 15  
TTT CGT ACT CAA GAA GAT GAA TAT GTT GCA ATA TTG GAT GCT TTA GAA 96  
Phe Arg Thr Gln Glu Asp Glu Tyr Val Ala Ile Leu Asp Ala Leu Glu  
20 25 30

GAA TAT CAT AAT ATG TCA GAG AAT ACT GTA GTC GAA AAA TAT TTA AAA	144
Glu Tyr His Asn Met Ser Glu Asn Thr Val Val Glu Lys Tyr Leu Lys	
35 40 45	
TTA AAA GAT ATA AAT AGT TTA ACA GAT ATT TAT ATA GAT ACA TAT AAA	192
Leu Lys Asp Ile Asn Ser Leu Thr Asp Ile Tyr Ile Asp Thr Tyr Lys	
50 55 60	
AAA TCT GGT AGA AAT AAA GCC TTA AAA AAA TTT AAG GAA TAT CTA GTT	240
Lys Ser Gly Arg Asn Lys Ala Leu Lys Lys Phe Lys Glu Tyr Leu Val	
65 70 75 80	
ACA GAA GTA TTA GAG CTA AAG AAT AAT AAT TTA ACT CCA GTT GAG AAA	288
Thr Glu Val Leu Glu Leu Lys Asn Asn Asn Leu Thr Pro Val Glu Lys	
85 90 95	
AAT TTA CAT TTT GTT TGG ATT GGA GGT CAA ATA AAT GAC ACT GCT ATT	336
Asn Leu His Phe Val Trp Ile Gly Gly Gln Ile Asn Asp Thr Ala Ile	
100 105 110	
AAT TAT ATA AAT CAA TGG AAA GAT GTA AAT AGT GAT TAT AAT GTT AAT	384
Asn Tyr Ile Asn Gln Trp Lys Asp Val Asn Ser Asp Tyr Asn Val Asn	
115 120 125	
GTT TTT TAT GAT AGT AAT GCA TTT TTG ATA AAC ACA TTG AAA AAA ACT	432
Val Phe Tyr Asp Ser Asn Ala Phe Leu Ile Asn Thr Leu Lys Lys Thr	
130 135 140	
GTA GTA GAA TCA GCA ATA AAT GAT ACA CTT GAA TCA TTT AGA GAA AAC	480
Val Val Glu Ser Ala Ile Asn Asp Thr Leu Glu Ser Phe Arg Glu Asn	
145 150 155 160	
TTA AAT GAC CCT AGA TTT GAC TAT AAT AAA TTC TTC AGA AAA CGT ATG	528
Leu Asn Asp Pro Arg Phe Asp Tyr Asn Lys Phe Phe Arg Lys Arg Met	
165 170 175	
GAA ATA ATT TAT GAT AAA CAG AAA AAT TTC ATA AAC TAC TAT AAA GCT	576
Glu Ile Ile Tyr Asp Lys Gln Lys Asn Phe Ile Asn Tyr Tyr Lys Ala	
180 185 190	
CAA AGA GAA GAA AAT CCT GAA CTT ATA ATT GAT GAT ATT GTA AAG ACA	624
Gln Arg Glu Glu Asn Pro Glu Leu Ile Ile Asp Asp Ile Val Lys Thr	
195 200 205	
TAT CTT TCA AAT GAG TAT TCA AAG GAG ATA GAT GAA CTT AAT ACC TAT	672
Tyr Leu Ser Asn Glu Tyr Ser Lys Glu Ile Asp Glu Leu Asn Thr Tyr	
210 215 220	
ATT GAA GAA TCC TTA AAT AAA ATT ACA CAG AAT AGT GGA AAT GAT GTT	720
Ile Glu Glu Ser Leu Asn Lys Ile Thr Gln Asn Ser Gly Asn Asp Val	
225 230 235 240	
AGA AAC TTT GAA GAA TTT AAA AAT GGA GAG TCA TTC AAC TTA TAT GAA	768
Arg Asn Phe Glu Glu Phe Lys Asn Gly Glu Ser Phe Asn Leu Tyr Glu	
245 250 255	
CAA GAG TTG GTA GAA AGG TGG AAT TTA GCT GCT GCT TCT GAC ATA TTA	816
Gln Glu Leu Val Glu Arg Trp Asn Leu Ala Ala Ala Ser Asp Ile Leu	
260 265 270	
AGA ATA TCT GCA TTA AAA GAA ATT GGT GGT ATG TAT TTA GAT GTT GAT	864
Arg Ile Ser Ala Leu Lys Glu Ile Gly Gly Met Tyr Leu Asp Val Asp	

275					280					285						
ATG	TTA	CCA	GGA	ATA	CAA	CCA	GAC	TTA	TTT	GAG	TCT	ATA	GAG	AAA	CCT	912
Met	Leu	Pro	Gly	Ile	Gln	Pro	Asp	Leu	Phe	Glu	Ser	Ile	Glu	Lys	Pro	
290					295					300						
AGT	TCA	GTA	ACA	GTG	GAT	TTT	TGG	GAA	ATG	ACA	AAG	TTA	GAA	GCT	ATA	960
Ser	Ser	Val	Thr	Val	Asp	Phe	Trp	Glu	Met	Thr	Lys	Leu	Glu	Ala	Ile	
305					310					315					320	
ATG	AAA	TAC	AAA	GAA	TAT	ATA	CCA	GAA	TAT	ACC	TCA	GAA	CAT	TTT	GAC	1008
Met	Lys	Tyr	Lys	Glu	Tyr	Ile	Pro	Glu	Tyr	Thr	Ser	Glu	His	Phe	Asp	
325					330					335						
ATG	TTA	GAC	GAA	GAA	GTT	CAA	AGT	AGT	TTT	GAA	TCT	GTT	CTA	GCT	TCT	1056
Met	Leu	Asp	Glu	Glu	Val	Gln	Ser	Ser	Phe	Glu	Ser	Val	Leu	Ala	Ser	
340					345					350						
AAG	TCA	GAT	AAA	TCA	GAA	ATA	TTC	TCA	TCA	CTT	GGT	GAT	ATG	GAG	GCA	1104
Lys	Ser	Asp	Lys	Ser	Glu	Ile	Phe	Ser	Ser	Leu	Gly	Asp	Met	Glu	Ala	
355					360					365						
TCA	CCA	CTA	GAA	GTT	AAA	ATT	GCA	TTT	AAT	AGT	AAG	GGT	ATT	ATA	AAT	1152
Ser	Pro	Leu	Glu	Val	Lys	Ile	Ala	Phe	Asn	Ser	Lys	Gly	Ile	Ile	Asn	
370					375					380						
CAA	GGG	CTA	ATT	TCT	GTG	AAA	GAC	TCA	TAT	TGT	AGC	AAT	TTA	ATA	GTA	1200
Gln	Gly	Leu	Ile	Ser	Val	Lys	Asp	Ser	Tyr	Cys	Ser	Asn	Leu	Ile	Val	
385					390					395					400	
AAA	CAA	ATC	GAG	AAT	AGA	TAT	AAA	ATA	TTG	AAT	AAT	AGT	TTA	AAT	CCA	1248
Lys	Gln	Ile	Glu	Asn	Arg	Tyr	Lys	Ile	Leu	Asn	Asn	Ser	Leu	Asn	Pro	
405					410					415						
GCT	ATT	AGC	GAG	GAT	AAT	GAT	TTT	AAT	ACT	ACA	ACG	AAT	ACC	TTT	ATT	1296
Ala	Ile	Ser	Glu	Asp	Asn	Asp	Phe	Asn	Thr	Thr	Thr	Asn	Thr	Phe	Ile	
420					425					430						
GAT	AGT	ATA	ATG	GCT	GAA	GCT	AAT	GCA	GAT	AAT	GGT	AGA	TTT	ATG	ATG	1344
Asp	Ser	Ile	Met	Ala	Glu	Ala	Asn	Ala	Asp	Asn	Gly	Arg	Phe	Met	Met	
435					440					445						
GAA	CTA	GGA	AAG	TAT	TTA	AGA	GTT	GGT	TTC	TTC	CCA	GAT	GTT	AAA	ACT	1392
Glu	Leu	Gly	Lys	Tyr	Leu	Arg	Val	Gly	Phe	Phe	Pro	Asp	Val	Lys	Thr	
450					455					460						
ACT	ATT	AAC	TTA	AGT	GGC	CCT	GAA	GCA	TAT	GCG	GCA	GCT	TAT	CAA	GAT	1440
Thr	Ile	Asn	Leu	Ser	Gly	Pro	Glu	Ala	Tyr	Ala	Ala	Ala	Tyr	Gln	Asp	
465					470					475					480	
TTA	TTA	ATG	TTT	AAA	GAA	GGC	AGT	ATG	AAT	ATC	CAT	TTG	ATA	GAA	GCT	1488
Leu	Leu	Met	Phe	Lys	Glu	Gly	Ser	Met	Asn	Ile	His	Leu	Ile	Glu	Ala	
485					490					495						
GAT	TTA	AGA	AAC	TTT	GAA	ATC	TCT	AAA	ACT	AAT	ATT	TCT	CAA	TCA	ACT	1536
Asp	Leu	Arg	Asn	Phe	Glu	Ile	Ser	Lys	Thr	Asn	Ile	Ser	Gln	Ser	Thr	
500					505					510						
GAA	CAA	GAA	ATG	GCT	AGC	TTA	TGG	TCA	TTT	GAC	GAT	GCA	AGA	GCT	AAA	1584
Glu	Gln	Glu	Met	Ala	Ser	Leu	Trp	Ser	Phe	Asp	Asp	Ala	Arg	Ala	Lys	
515					520					525						

GCT CAA TTT GAA GAA TAT AAA AGG AAT TAT TTT GAA GGT TCT CTT GGT Ala Gln Phe Glu Glu Tyr Lys Arg Asn Tyr Phe Glu Gly Ser Leu Gly 530 535 540	1632
GAA GAT GAT AAT CTT GAT TTT TCT CAA AAT ATA GTA GTT GAC AAG GAG Glu Asp Asp Asn Leu Asp Phe Ser Gln Asn Ile Val Val Asp Lys Glu 545 550 555 560	1680
TAT CTT TTA GAA AAA ATA TCT TCA TTA GCA AGA AGT TCA GAG AGA GGA Tyr Leu Leu Glu Lys Ile Ser Ser Leu Ala Arg Ser Ser Glu Arg Gly 565 570 575	1728
TAT ATA CAC TAT ATT GTT CAG TTA CAA GGA GAT AAA ATT AGT TAT GAA Tyr Ile His Tyr Ile Val Gln Leu Gln Gly Asp Lys Ile Ser Tyr Glu 580 585 590	1776
GCA GCA TGT AAC TTA TTT GCA AAG ACT CCT TAT GAT AGT GTA CTG TTT Ala Ala Cys Asn Leu Phe Ala Lys Thr Pro Tyr Asp Ser Val Leu Phe 595 600 605	1824
CAG AAA AAT ATA GAA GAT TCA GAA ATT GCA TAT TAT TAT AAT CCT GGA Gln Lys Asn Ile Glu Asp Ser Glu Ile Ala Tyr Tyr Tyr Asn Pro Gly 610 615 620	1872
GAT GGT GAA ATA CAA GAA ATA GAC AAG TAT AAA ATT CCA AGT ATA ATT Asp Gly Glu Ile Gln Glu Ile Asp Lys Tyr Lys Ile Pro Ser Ile Ile 625 630 635 640	1920
TCT GAT AGA CCT AAG ATT AAA TTA ACA TTT ATT GGT CAT GGT AAA GAT Ser Asp Arg Pro Lys Ile Lys Leu Thr Phe Ile Gly His Gly Lys Asp 645 650 655	1968
GAA TTT AAT ACT GAT ATA TTT GCA GGT TTT GAT GTA GAT TCA TTA TCC Glu Phe Asn Thr Asp Ile Phe Ala Gly Phe Asp Val Asp Ser Leu Ser 660 665 670	2016
ACA GAA ATA GAA GCA GCA ATA GAT TTA GCT AAA GAG GAT ATT TCT CCT Thr Glu Ile Glu Ala Ala Ile Asp Leu Ala Lys Glu Asp Ile Ser Pro 675 680 685	2064
AAG TCA ATA GAA ATA AAT TTA TTA GGA TGT AAT ATG TTT AGC TAC TCT Lys Ser Ile Glu Ile Asn Leu Leu Gly Cys Asn Met Phe Ser Tyr Ser 690 695 700	2112
ATC AAC GTA GAG GAG ACT TAT CCT GGA AAA TTA TTA CTT AAA GTT AAA Ile Asn Val Glu Glu Thr Tyr Pro Gly Lys Leu Leu Leu Lys Val Lys 705 710 715 720	2160
GAT AAA ATA TCA GAA TTA ATG CCA TCT ATA AGT CAA GAC TCT ATT ATA Asp Lys Ile Ser Glu Leu Met Pro Ser Ile Ser Gln Asp Ser Ile Ile 725 730 735	2208
GTA AGT GCA AAT CAA TAT GAA GTT AGA ATA AAT AGT GAA GGA AGA AGA Val Ser Ala Asn Gln Tyr Glu Val Arg Ile Asn Ser Glu Gly Arg Arg 740 745 750	2256
GAA TTA TTG GAT CAT TCT GGT GAA TGG ATA AAT AAA GAA GAA AGT ATT Glu Leu Leu Asp His Ser Gly Glu Trp Ile Asn Lys Glu Glu Ser Ile 755 760 765	2304

ATA AAG GAT ATT TCA TCA AAA GAA TAT ATA TCA TTT AAT CCT AAA GAA Ile Lys Asp Ile Ser Ser Lys Glu Tyr Ile Ser Phe Asn Pro Lys Glu 770 775 780	2352
AAT AAA ATT ACA GTA AAA TCT AAA AAT TTA CCT GAG CTA TCT ACA TTA Asn Lys Ile Thr Val Lys Ser Lys Asn Leu Pro Glu Leu Ser Thr Leu 785 790 795 800	2400
TTA CAA GAA ATT AGA AAT AAT TCT AAT TCA AGT GAT ATT GAA CTA GAA Leu Gln Glu Ile Arg Asn Asn Ser Asn Ser Ser Asp Ile Glu Leu Glu 805 810 815	2448
GAA AAA GTA ATG TTA ACA GAA TGT GAG ATA AAT GTT ATT TCA AAT ATA Glu Lys Val Met Leu Thr Glu Cys Glu Ile Asn Val Ile Ser Asn Ile 820 825 830	2496
GAT ACG CAA ATT GTT GAG GAA AGG ATT GAA GAA GCT AAG AAT TTA ACT Asp Thr Gln Ile Val Glu Glu Arg Ile Glu Glu Ala Lys Asn Leu Thr 835 840 845	2544
TCT GAC TCT ATT AAT TAT ATA AAA GAT GAA TTT AAA CTA ATA GAA TCT Ser Asp Ser Ile Asn Tyr Ile Lys Asp Glu Phe Lys Leu Ile Glu Ser 850 855 860	2592
ATT TCT GAT GCA CTA TGT GAC TTA AAA CAA CAG AAT GAA TTA GAA GAT Ile Ser Asp Ala Leu Cys Asp Leu Lys Gln Gln Asn Glu Leu Glu Asp 865 870 875 880	2640
TCT CAT TTT ATA TCT TTT GAG GAC ATA TCA GAG ACT GAT GAG GGA TTT Ser His Phe Ile Ser Phe Glu Asp Ile Ser Glu Thr Asp Glu Gly Phe 885 890 895	2688
AGT ATA AGA TTT ATT AAT AAA GAA ACT GGA GAA TCT ATA TTT GTA GAA Ser Ile Arg Phe Ile Asn Lys Glu Thr Gly Glu Ser Ile Phe Val Glu 900 905 910	2736
ACT GAA AAA ACA ATA TTC TCT GAA TAT GCT AAT CAT ATA ACT GAA GAG Thr Glu Lys Thr Ile Phe Ser Glu Tyr Ala Asn His Ile Thr Glu Glu 915 920 925	2784
ATT TCT AAG ATA AAA GGT ACT ATA TTT GAT ACT GTA AAT GGT AAG TTA Ile Ser Lys Ile Lys Gly Thr Ile Phe Asp Thr Val Asn Gly Lys Leu 930 935 940	2832
GTA AAA AAA GTA AAT TTA GAT ACT ACA CAC GAA GTA AAT ACT TTA AAT Val Lys Lys Val Asn Leu Asp Thr Thr His Glu Val Asn Thr Leu Asn 945 950 955 960	2880
GCT GCA TTT TTT ATA CAA TCA TTA ATA GAA TAT AAT AGT TCT AAA GAA Ala Ala Phe Phe Ile Gln Ser Leu Ile Glu Tyr Asn Ser Ser Lys Glu 965 970 975	2928
TCT CTT AGT AAT TTA AGT GTA GCA ATG AAA GTC CAA GTT TAC GCT CAA Ser Leu Ser Asn Leu Ser Val Ala Met Lys Val Gln Val Tyr Ala Gln 980 985 990	2976
TTA TTT AGT ACT GGT TTA AAT ACT ATT ACA GAT GCA GCC AAA GTT GTT Leu Phe Ser Thr Gly Leu Asn Thr Ile Thr Asp Ala Ala Lys Val Val 995 1000 1005	3024



GAA TTA GTA TCA ACT GCA TTA GAT GAA ACT ATA GAC TTA CTT CCT ACA Glu Leu Val Ser Thr Ala Leu Asp Glu Thr Ile Asp Leu Leu Pro Thr 1010 1015 1020	3072
TTA TCT GAA GGA TTA CCT ATA ATT GCA ACT ATT ATA GAT GGT GTA AGT Leu Ser Glu Gly Leu Pro Ile Ile Ala Thr Ile Ile Asp Gly Val Ser 1025 1030 1035 1040	3120
TTA GGT GCA GCA ATC AAA GAG CTA AGT GAA ACG AGT GAC CCA TTA TTA Leu Gly Ala Ala Ile Lys Glu Leu Ser Glu Thr Ser Asp Pro Leu Leu 1045 1050 1055	3168
AGA CAA GAA ATA GAA GCT AAG ATA GGT ATA ATG GCA GTA AAT TTA ACA Arg Gln Glu Ile Glu Ala Lys Ile Gly Ile Met Ala Val Asn Leu Thr 1060 1065 1070	3216
ACA GCT ACA ACT GCA ATC ATT ACT TCA TCT TTG GGG ATA GCT AGT GGA Thr Ala Thr Thr Ala Ile Ile Thr Ser Ser Leu Gly Ile Ala Ser Gly 1075 1080 1085	3264
TTT AGT ATA CTT TTA GTT CCT TTA GCA GGA ATT TCA GCA GGT ATA CCA Phe Ser Ile Leu Leu Val Pro Leu Ala Gly Ile Ser Ala Gly Ile Pro 1090 1095 1100	3312
AGC TTA GTA AAC AAT GAA CTT GTA CTT CGA GAT AAG GCA ACA AAG GTT Ser Leu Val Asn Asn Glu Leu Val Leu Arg Asp Lys Ala Thr Lys Val 1105 1110 1115 1120	3360
GTA GAT TAT TTT AAA CAT GTT TCA TTA GTT GAA ACT GAA GGA GTA TTT Val Asp Tyr Phe Lys His Val Ser Leu Val Glu Thr Glu Gly Val Phe 1125 1130 1135	3408
ACT TTA TTA GAT GAT AAA ATA ATG ATG CCA CAA GAT GAT TTA GTG ATA Thr Leu Leu Asp Asp Lys Ile Met Met Pro Gln Asp Asp Leu Val Ile 1140 1145 1150	3456
TCA GAA ATA GAT TTT AAT AAT AAT TCA ATA GTT TTA GGT AAA TGT GAA Ser Glu Ile Asp Phe Asn Asn Asn Ser Ile Val Leu Gly Lys Cys Glu 1155 1160 1165	3504
ATC TGG AGA ATG GAA GGT GGT TCA GGT CAT ACT GTA ACT GAT GAT ATA Ile Trp Arg Met Glu Gly Gly Ser Gly His Thr Val Thr Asp Asp Ile 1170 1175 1180	3552
GAT CAC TTC TTT TCA GCA CCA TCA ATA ACA TAT AGA GAG CCA CAC TTA Asp His Phe Phe Ser Ala Pro Ser Ile Thr Tyr Arg Glu Pro His Leu 1185 1190 1195 1200	3600
TCT ATA TAT GAC GTA TTG GAA GTA CAA AAA GAA GAA CTT GAT TTG TCA Ser Ile Tyr Asp Val Leu Glu Val Gln Lys Glu Glu Leu Asp Leu Ser 1205 1210 1215	3648
AAA GAT TTA ATG GTA TTA CCT AAT GCT CCA AAT AGA GTA TTT GCT TGG Lys Asp Leu Met Val Leu Pro Asn Ala Pro Asn Arg Val Phe Ala Trp 1220 1225 1230	3696
GAA ACA GGA TGG ACA CCA GGT TTA AGA AGC TTA GAA AAT GAT GGC ACA Glu Thr Gly Trp Thr Pro Gly Leu Arg Ser Leu Glu Asn Asp Gly Thr 1235 1240 1245	3744

AAA CTG TTA GAC CGT ATA AGA GAT AAC TAT GAA GGT GAG TTT TAT TGG Lys Leu Leu Asp Arg Ile Arg Asp Asn Tyr Glu Gly Glu Phe Tyr Trp 1250 1255 1260	3792
AGA TAT TTT GCT TTT ATA GCT GAT GCT TTA ATA ACA ACA TTA AAA CCA Arg Tyr Phe Ala Phe Ile Ala Asp Ala Leu Ile Thr Thr Leu Lys Pro 1265 1270 1275 1280	3840
AGA TAT GAA GAT ACT AAT ATA AGA ATA AAT TTA GAT AGT AAT ACT AGA Arg Tyr Glu Asp Thr Asn Ile Arg Ile Asn Leu Asp Ser Asn Thr Arg 1285 1290 1295	3888
AGT TTT ATA GTT CCA ATA ATA ACT ACA GAA TAT ATA AGA GAA AAA TTA Ser Phe Ile Val Pro Ile Ile Thr Thr Glu Tyr Ile Arg Glu Lys Leu 1300 1305 1310	3936
TCA TAT TCT TTC TAT GGT TCA GGA GGA ACT TAT GCA TTG TCT CTT TCT Ser Tyr Ser Phe Tyr Gly Ser Gly Gly Thr Tyr Ala Leu Ser Leu Ser 1315 1320 1325	3984
CAA TAT AAT ATG GGT ATA AAT ATA GAA TTA AGT GAA AGT GAT GTT TGG Gln Tyr Asn Met Gly Ile Asn Ile Glu Leu Ser Glu Ser Asp Val Trp 1330 1335 1340	4032
ATT ATA GAT GTT GAT AAT GTT GTG AGA GAT GTA ACT ATA GAA TCT GAT Ile Ile Asp Val Asp Asn Val Val Arg Asp Val Thr Ile Glu Ser Asp 1345 1350 1355 1360	4080
AAA ATT AAA AAA GGT GAT TTA ATA GAA GGT ATT TTA TCT ACA CTA AGT Lys Ile Lys Lys Gly Asp Leu Ile Glu Gly Ile Leu Ser Thr Leu Ser 1365 1370 1375	4128
ATT GAA GAG AAT AAA ATT ATC TTA AAT AGC CAT GAG ATT AAT TTT TCT Ile Glu Glu Asn Lys Ile Ile Leu Asn Ser His Glu Ile Asn Phe Ser 1380 1385 1390	4176
GGT GAG GTA AAT GGA AGT AAT GGA TTT GTT TCT TTA ACA TTT TCA ATT Gly Glu Val Asn Gly Ser Asn Gly Phe Val Ser Leu Thr Phe Ser Ile 1395 1400 1405	4224
TTA GAA GGA ATA AAT GCA ATT ATA GAA GTT GAT TTA TTA TCT AAA TCA Leu Glu Gly Ile Asn Ala Ile Ile Glu Val Asp Leu Leu Ser Lys Ser 1410 1415 1420	4272
TAT AAA TTA CTT ATT TCT GGC GAA TTA AAA ATA TTG ATG TTA AAT TCA Tyr Lys Leu Leu Ile Ser Gly Glu Leu Lys Ile Leu Met Leu Asn Ser 1425 1430 1435 1440	4320
AAT CAT ATT CAA CAG AAA ATA GAT TAT ATA GGA TTC AAT AGC GAA TTA Asn His Ile Gln Gln Lys Ile Asp Tyr Ile Gly Phe Asn Ser Glu Leu 1445 1450 1455	4368
CAG AAA AAT ATA CCA TAT AGC TTT GTA GAT AGT GAA GGA AAA GAG AAT Gln Lys Asn Ile Pro Tyr Ser Phe Val Asp Ser Glu Gly Lys Glu Asn 1460 1465 1470	4416
GGT TTT ATT AAT GGT TCA ACA AAA GAA GGT TTA TTT GTA TCT GAA TTA Gly Phe Ile Asn Gly Ser Thr Lys Glu Gly Leu Phe Val Ser Glu Leu 1475 1480 1485	4464

CCT GAT GTA GTT CTT ATA AGT AAG GTT TAT ATG GAT GAT AGT AAG CCT Pro Asp Val Val Leu Ile Ser Lys Val Tyr Met Asp Asp Ser Lys Pro 1490 1495 1500	4512
TCA TTT GGA TAT TAT AGT AAT AAT TTG AAA GAT GTC AAA GTT ATA ACT Ser Phe Gly Tyr Tyr Ser Asn Asn Leu Lys Asp Val Lys Val Ile Thr 1505 1510 1515 1520	4560
AAA GAT AAT GTT AAT ATA TTA ACA GGT TAT TAT CTT AAG GAT GAT ATA Lys Asp Asn Val Asn Ile Leu Thr Gly Tyr Tyr Leu Lys Asp Asp Ile 1525 1530 1535	4608
AAA ATC TCT CTT TCT TTG ACT CTA CAA GAT GAA AAA ACT ATA AAG TTA Lys Ile Ser Leu Ser Leu Thr Leu Gln Asp Glu Lys Thr Ile Lys Leu 1540 1545 1550	4656
AAT AGT GTG CAT TTA GAT GAA AGT GGA GTA GCT GAG ATT TTG AAG TTC Asn Ser Val His Leu Asp Glu Ser Gly Val Ala Glu Ile Leu Lys Phe 1555 1560 1565	4704
ATG AAT AGA AAA GGT AAT ACA AAT ACT TCA GAT TCT TTA ATG AGC TTT Met Asn Arg Lys Gly Asn Thr Asn Thr Ser Asp Ser Leu Met Ser Phe 1570 1575 1580	4752
TTA GAA AGT ATG AAT ATA AAA AGT ATT TTC GTT AAT TTC TTA CAA TCT Leu Glu Ser Met Asn Ile Lys Ser Ile Phe Val Asn Phe Leu Gln Ser 1585 1590 1595 1600	4800
AAT ATT AAG TTT ATA TTA GAT GCT AAT TTT ATA ATA AGT GGT ACT ACT Asn Ile Lys Phe Ile Leu Asp Ala Asn Phe Ile Ile Ser Gly Thr Thr 1605 1610 1615	4848
TCT ATT GGC CAA TTT GAG TTT ATT TGT GAT GAA AAT GAT AAT ATA CAA Ser Ile Gly Gln Phe Glu Phe Ile Cys Asp Glu Asn Asp Asn Ile Gln 1620 1625 1630	4896
CCA TAT TTC ATT AAG TTT AAT ACA CTA GAA ACT AAT TAT ACT TTA TAT Pro Tyr Phe Ile Lys Phe Asn Thr Leu Glu Thr Asn Tyr Thr Leu Tyr 1635 1640 1645	4944
GTA GGA AAT AGA CAA AAT ATG ATA GTG GAA CCA AAT TAT GAT TTA GAT Val Gly Asn Arg Gln Asn Met Ile Val Glu Pro Asn Tyr Asp Leu Asp 1650 1655 1660	4992
GAT TCT GGA GAT ATA TCT TCA ACT GTT ATC AAT TTC TCT CAA AAG TAT Asp Ser Gly Asp Ile Ser Ser Thr Val Ile Asn Phe Ser Gln Lys Tyr 1665 1670 1675 1680	5040
CTT TAT GGA ATA GAC AGT TGT GTT AAT AAA GTT GTA ATT TCA CCA AAT Leu Tyr Gly Ile Asp Ser Cys Val Asn Lys Val Val Ile Ser Pro Asn 1685 1690 1695	5088
ATT TAT ACA GAT GAA ATA AAT ATA ACG CCT GTA TAT GAA ACA AAT AAT Ile Tyr Thr Asp Glu Ile Asn Ile Thr Pro Val Tyr Glu Thr Asn Asn 1700 1705 1710	5136
ACT TAT CCA GAA GTT ATT GTA TTA GAT GCA AAT TAT ATA AAT GAA AAA Thr Tyr Pro Glu Val Ile Val Leu Asp Ala Asn Tyr Ile Asn Glu Lys 1715 1720 1725	5184

ATA AAT GTT AAT ATC AAT GAT CTA TCT ATA CGA TAT GTA TGG AGT AAT Ile Asn Val Asn Ile Asn Asp Leu Ser Ile Arg Tyr Val Trp Ser Asn 1730 1735 1740	5232
GAT GGT AAT GAT TTT ATT CTT ATG TCA ACT AGT GAA GAA AAT AAG GTG Asp Gly Asn Asp Phe Ile Leu Met Ser Thr Ser Glu Glu Asn Lys Val 1745 1750 1755 1760	5280
TCA CAA GTT AAA ATA AGA TTC GTT AAT GTT TTT AAA GAT AAG ACT TTG Ser Gln Val Lys Ile Arg Phe Val Asn Val Phe Lys Asp Lys Thr Leu 1765 1770 1775	5328
GCA AAT AAG CTA TCT TTT AAC TTT AGT GAT AAA CAA GAT GTA CCT GTA Ala Asn Lys Leu Ser Phe Asn Phe Ser Asp Lys Gln Asp Val Pro Val 1780 1785 1790	5376
AGT GAA ATA ATC TTA TCA TTT ACA CCT TCA TAT TAT GAG GAT GGA TTG Ser Glu Ile Ile Leu Ser Phe Thr Pro Ser Tyr Tyr Glu Asp Gly Leu 1795 1800 1805	5424
ATT GGC TAT GAT TTG GGT CTA GTT TCT TTA TAT AAT GAG AAA TTT TAT Ile Gly Tyr Asp Leu Gly Leu Val Ser Leu Tyr Asn Glu Lys Phe Tyr 1810 1815 1820	5472
ATT AAT AAC TTT GGA ATG ATG GTA TCT GGA TTA ATA TAT ATT AAT GAT Ile Asn Asn Phe Gly Met Met Val Ser Gly Leu Ile Tyr Ile Asn Asp 1825 1830 1835 1840	5520
TCA TTA TAT TAT TTT AAA CCA CCA GTA AAT AAT TTG ATA ACT GGA TTT Ser Leu Tyr Tyr Phe Lys Pro Pro Val Asn Asn Leu Ile Thr Gly Phe 1845 1850 1855	5568
GTG ACT GTA GGC GAT GAT AAA TAC TAC TTT AAT CCA ATT AAT GGT GGA Val Thr Val Gly Asp Asp Lys Tyr Tyr Phe Asn Pro Ile Asn Gly Gly 1860 1865 1870	5616
GCT GCT TCA ATT GGA GAG ACA ATA ATT GAT GAC AAA AAT TAT TAT TTC Ala Ala Ser Ile Gly Glu Thr Ile Ile Asp Asp Lys Asn Tyr Tyr Phe 1875 1880 1885	5664
AAC CAA AGT GGA GTG TTA CAA ACA GGT GTA TTT AGT ACA GAA GAT GGA Asn Gln Ser Gly Val Leu Gln Thr Gly Val Phe Ser Thr Glu Asp Gly 1890 1895 1900	5712
TTT AAA TAT TTT GCC CCA GCT AAT ACA CTT GAT GAA AAC CTA GAA GGA Phe Lys Tyr Phe Ala Pro Ala Asn Thr Leu Asp Glu Asn Leu Glu Gly 1905 1910 1915 1920	5760
GAA GCA ATT GAT TTT ACT GGA AAA TTA ATT ATT GAC GAA AAT ATT TAT Glu Ala Ile Asp Phe Thr Gly Lys Leu Ile Ile Asp Glu Asn Ile Tyr 1925 1930 1935	5808
TAT TTT GAT GAT AAT TAT AGA GGA GCT GTA GAA TGG AAA GAA TTA GAT Tyr Phe Asp Asp Asn Tyr Arg Gly Ala Val Glu Trp Lys Glu Leu Asp 1940 1945 1950	5856
GGT GAA ATG CAC TAT TTT AGC CCA GAA ACA GGT AAA GCT TTT AAA GGT Gly Glu Met His Tyr Phe Ser Pro Glu Thr Gly Lys Ala Phe Lys Gly 1955 1960 1965	5904

CTA AAT CAA ATA GGT GAT TAT AAA TAC TAT TTC AAT TCT GAT GGA GTT Leu Asn Gln Ile Gly Asp Tyr Lys Tyr Tyr Phe Asn Ser Asp Gly Val 1970 1975 1980	5952
ATG CAA AAA GGA TTT GTT AGT ATA AAT GAT AAT AAA CAC TAT TTT GAT Met Gln Lys Gly Phe Val Ser Ile Asn Asp Asn Lys His Tyr Phe Asp 1985 1990 1995 2000	6000
GAT TCT GGT GTT ATG AAA GTA GGT TAC ACT GAA ATA GAT GGC AAG CAT Asp Ser Gly Val Met Lys Val Gly Tyr Thr Glu Ile Asp Gly Lys His 2005 2010 2015	6048
TTC TAC TTT GCT GAA AAC GGA GAA ATG CAA ATA GGA GTA TTT AAT ACA Phe Tyr Phe Ala Glu Asn Gly Glu Met Gln Ile Gly Val Phe Asn Thr 2020 2025 2030	6096
GAA GAT GGA TTT AAA TAT TTT GCT CAT CAT AAT GAA GAT TTA GGA AAT Glu Asp Gly Phe Lys Tyr Phe Ala His His Asn Glu Asp Leu Gly Asn 2035 2040 2045	6144
GAA GAA GGT GAA GAA ATC TCA TAT TCT GGT ATA TTA AAT TTC AAT AAT Glu Glu Gly Glu Glu Ile Ser Tyr Ser Gly Ile Leu Asn Phe Asn Asn 2050 2055 2060	6192
AAA ATT TAC TAT TTT GAT GAT TCA TTT ACA GCT GTA GTT GGA TGG AAA Lys Ile Tyr Tyr Phe Asp Asp Ser Phe Thr Ala Val Val Gly Trp Lys 2065 2070 2075 2080	6240
GAT TTA GAG GAT GGT TCA AAG TAT TAT TTT GAT GAA GAT ACA GCA GAA Asp Leu Glu Asp Gly Ser Lys Tyr Tyr Phe Asp Glu Asp Thr Ala Glu 2085 2090 2095	6288
GCA TAT ATA GGT TTG TCA TTA ATA AAT GAT GGT CAA TAT TAT TTT AAT Ala Tyr Ile Gly Leu Ser Leu Ile Asn Asp Gly Gln Tyr Tyr Phe Asn 2100 2105 2110	6336
GAT GAT GGA ATT ATG CAA GTT GGA TTT GTC ACT ATA AAT GAT AAA GTC Asp Asp Gly Ile Met Gln Val Gly Phe Val Thr Ile Asn Asp Lys Val 2115 2120 2125	6384
TTC TAC TTC TCT GAC TCT GGA ATT ATA GAA TCT GGA GTA CAA AAC ATA Phe Tyr Phe Ser Asp Ser Gly Ile Ile Glu Ser Gly Val Gln Asn Ile 2130 2135 2140	6432
GAT GAC AAT TAT TTC TAT ATA GAT GAT AAT GGT ATA GTT CAA ATT GGT Asp Asp Asn Tyr Phe Tyr Ile Asp Asp Asn Gly Ile Val Gln Ile Gly 2145 2150 2155 2160	6480
GTA TTT GAT ACT TCA GAT GGA TAT AAA TAT TTT GCA CCT GCT AAT ACT Val Phe Asp Thr Ser Asp Gly Tyr Lys Tyr Phe Ala Pro Ala Asn Thr 2165 2170 2175	6528
GTA AAT GAT AAT ATT TAC GGA CAA GCA GTT GAA TAT AGT GGT TTA GTT Val Asn Asp Asn Ile Tyr Gly Gln Ala Val Glu Tyr Ser Gly Leu Val 2180 2185 2190	6576
AGA GTT GGG GAA GAT GTA TAT TAT TTT GGA GAA ACA TAT ACA ATT GAG Arg Val Gly Glu Asp Val Tyr Tyr Phe Gly Glu Thr Tyr Thr Ile Glu 2195 2200 2205	6624

ACT GGA TGG ATA TAT GAT ATG GAA AAT GAA AGT GAT AAA TAT TAT TTC Thr Gly Trp Ile Tyr Asp Met Glu Asn Glu Ser Asp Lys Tyr Tyr Phe 2210 2215 2220	6672
AAT CCA GAA ACT AAA AAA GCA TGC AAA GGT ATT AAT TTA ATT GAT GAT Asn Pro Glu Thr Lys Lys Ala Cys Lys Gly Ile Asn Leu Ile Asp Asp 2225 2230 2235 2240	6720
ATA AAA TAT TAT TTT GAT GAG AAG GGC ATA ATG AGA ACG GGT CTT ATA Ile Lys Tyr Tyr Phe Asp Glu Lys Gly Ile Met Arg Thr Gly Leu Ile 2245 2250 2255	6768
TCA TTT GAA AAT AAT AAT TAT TAC TTT AAT GAG AAT GGT GAA ATG CAA Ser Phe Glu Asn Asn Asn Tyr Tyr Phe Asn Glu Asn Gly Glu Met Gln 2260 2265 2270	6816
TTT GGT TAT ATA AAT ATA GAA GAT AAG ATG TTC TAT TTT GGT GAA GAT Phe Gly Tyr Ile Asn Ile Glu Asp Lys Met Phe Tyr Phe Gly Glu Asp 2275 2280 2285	6864
GGT GTC ATG CAG ATT GGA GTA TTT AAT ACA CCA GAT GGA TTT AAA TAC Gly Val Met Gln Ile Gly Val Phe Asn Thr Pro Asp Gly Phe Lys Tyr 2290 2295 2300	6912
TTT GCA CAT CAA AAT ACT TTG GAT GAG AAT TTT GAG GGA GAA TCA ATA Phe Ala His Gln Asn Thr Leu Asp Glu Asn Phe Glu Gly Glu Ser Ile 2305 2310 2315 2320	6960
AAC TAT ACT GGT TGG TTA GAT TTA GAT GAA AAG AGA TAT TAT TTT ACA Asn Tyr Thr Gly Trp Leu Asp Leu Asp Glu Lys Arg Tyr Tyr Phe Thr 2325 2330 2335	7008
GAT GAA TAT ATT GCA GCA ACT GGT TCA GTT ATT ATT GAT GGT GAG GAG Asp Glu Tyr Ile Ala Ala Thr Gly Ser Val Ile Ile Asp Gly Glu Glu 2340 2345 2350	7056
TAT TAT TTT GAT CCT GAT ACA GCT CAA TTA GTG ATT AGT GAA Tyr Tyr Phe Asp Pro Asp Thr Ala Gln Leu Val Ile Ser Glu 2355 2360 2365	7098
TAG	7101

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2366 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Ser Leu Val Asn Arg Lys Gln Leu Glu Lys Met Ala Asn Val Arg 1 5 10 15
Phe Arg Thr Gln Glu Asp Glu Tyr Val Ala Ile Leu Asp Ala Leu Glu 20 25 30
Glu Tyr His Asn Met Ser Glu Asn Thr Val Val Glu Lys Tyr Leu Lys 35 40 45

Leu Lys Asp Ile Asn Ser Leu Thr Asp Ile Tyr Ile Asp Thr Tyr Lys  
 50 55 60  
 Lys Ser Gly Arg Asn Lys Ala Leu Lys Lys Phe Lys Glu Tyr Leu Val  
 65 70 75 80  
 Thr Glu Val Leu Glu Leu Lys Asn Asn Asn Leu Thr Pro Val Glu Lys  
 85 90 95  
 Asn Leu His Phe Val Trp Ile Gly Gly Gln Ile Asn Asp Thr Ala Ile  
 100 105 110  
 Asn Tyr Ile Asn Gln Trp Lys Asp Val Asn Ser Asp Tyr Asn Val Asn  
 115 120 125  
 Val Phe Tyr Asp Ser Asn Ala Phe Leu Ile Asn Thr Leu Lys Lys Thr  
 130 135 140  
 Val Val Glu Ser Ala Ile Asn Asp Thr Leu Glu Ser Phe Arg Glu Asn  
 145 150 155 160  
 Leu Asn Asp Pro Arg Phe Asp Tyr Asn Lys Phe Phe Arg Lys Arg Met  
 165 170 175  
 Glu Ile Ile Tyr Asp Lys Gln Lys Asn Phe Ile Asn Tyr Tyr Lys Ala  
 180 185 190  
 Gln Arg Glu Glu Asn Pro Glu Leu Ile Ile Asp Asp Ile Val Lys Thr  
 195 200 205  
 Tyr Leu Ser Asn Glu Tyr Ser Lys Glu Ile Asp Glu Leu Asn Thr Tyr  
 210 215 220  
 Ile Glu Glu Ser Leu Asn Lys Ile Thr Gln Asn Ser Gly Asn Asp Val  
 225 230 235 240  
 Arg Asn Phe Glu Glu Phe Lys Asn Gly Glu Ser Phe Asn Leu Tyr Glu  
 245 250 255  
 Gln Glu Leu Val Glu Arg Trp Asn Leu Ala Ala Ala Ser Asp Ile Leu  
 260 265 270  
 Arg Ile Ser Ala Leu Lys Glu Ile Gly Gly Met Tyr Leu Asp Val Asp  
 275 280 285  
 Met Leu Pro Gly Ile Gln Pro Asp Leu Phe Glu Ser Ile Glu Lys Pro  
 290 295 300  
 Ser Ser Val Thr Val Asp Phe Trp Glu Met Thr Lys Leu Glu Ala Ile  
 305 310 315 320  
 Met Lys Tyr Lys Glu Tyr Ile Pro Glu Tyr Thr Ser Glu His Phe Asp  
 325 330 335  
 Met Leu Asp Glu Glu Val Gln Ser Ser Phe Glu Ser Val Leu Ala Ser  
 340 345 350  
 Lys Ser Asp Lys Ser Glu Ile Phe Ser Ser Leu Gly Asp Met Glu Ala  
 355 360 365  
 Ser Pro Leu Glu Val Lys Ile Ala Phe Asn Ser Lys Gly Ile Ile Asn  
 370 375 380

Gln	Gly	Leu	Ile	Ser	Val	Lys	Asp	Ser	Tyr	Cys	Ser	Asn	Leu	Ile	Val
385					390					395					400
Lys	Gln	Ile	Glu	Asn	Arg	Tyr	Lys	Ile	Leu	Asn	Asn	Ser	Leu	Asn	Pro
				405					410					415	
Ala	Ile	Ser	Glu	Asp	Asn	Asp	Phe	Asn	Thr	Thr	Thr	Asn	Thr	Phe	Ile
			420					425					430		
Asp	Ser	Ile	Met	Ala	Glu	Ala	Asn	Ala	Asp	Asn	Gly	Arg	Phe	Met	Met
		435					440					445			
Glu	Leu	Gly	Lys	Tyr	Leu	Arg	Val	Gly	Phe	Phe	Pro	Asp	Val	Lys	Thr
	450					455					460				
Thr	Ile	Asn	Leu	Ser	Gly	Pro	Glu	Ala	Tyr	Ala	Ala	Ala	Tyr	Gln	Asp
465					470					475					480
Leu	Leu	Met	Phe	Lys	Glu	Gly	Ser	Met	Asn	Ile	His	Leu	Ile	Glu	Ala
				485					490					495	
Asp	Leu	Arg	Asn	Phe	Glu	Ile	Ser	Lys	Thr	Asn	Ile	Ser	Gln	Ser	Thr
			500					505					510		
Glu	Gln	Glu	Met	Ala	Ser	Leu	Trp	Ser	Phe	Asp	Asp	Ala	Arg	Ala	Lys
		515					520					525			
Ala	Gln	Phe	Glu	Glu	Tyr	Lys	Arg	Asn	Tyr	Phe	Glu	Gly	Ser	Leu	Gly
	530					535					540				
Glu	Asp	Asp	Asn	Leu	Asp	Phe	Ser	Gln	Asn	Ile	Val	Val	Asp	Lys	Glu
545					550					555					560
Tyr	Leu	Leu	Glu	Lys	Ile	Ser	Ser	Leu	Ala	Arg	Ser	Ser	Glu	Arg	Gly
				565					570					575	
Tyr	Ile	His	Tyr	Ile	Val	Gln	Leu	Gln	Gly	Asp	Lys	Ile	Ser	Tyr	Glu
			580					585					590		
Ala	Ala	Cys	Asn	Leu	Phe	Ala	Lys	Thr	Pro	Tyr	Asp	Ser	Val	Leu	Phe
		595					600					605			
Gln	Lys	Asn	Ile	Glu	Asp	Ser	Glu	Ile	Ala	Tyr	Tyr	Tyr	Asn	Pro	Gly
	610					615					620				
Asp	Gly	Glu	Ile	Gln	Glu	Ile	Asp	Lys	Tyr	Lys	Ile	Pro	Ser	Ile	Ile
625				630						635					640
Ser	Asp	Arg	Pro	Lys	Ile	Lys	Leu	Thr	Phe	Ile	Gly	His	Gly	Lys	Asp
				645					650					655	
Glu	Phe	Asn	Thr	Asp	Ile	Phe	Ala	Gly	Phe	Asp	Val	Asp	Ser	Leu	Ser
			660					665					670		
Thr	Glu	Ile	Glu	Ala	Ala	Ile	Asp	Leu	Ala	Lys	Glu	Asp	Ile	Ser	Pro
			675				680					685			
Lys	Ser	Ile	Glu	Ile	Asn	Leu	Leu	Gly	Cys	Asn	Met	Phe	Ser	Tyr	Ser
	690					695					700				
Ile	Asn	Val	Glu	Glu	Thr	Tyr	Pro	Gly	Lys	Leu	Leu	Leu	Lys	Val	Lys
705					710					715					720



Asp Lys Ile Ser Glu Leu Met Pro Ser Ile Ser Gln Asp Ser Ile Ile  
 725 730 735  
 Val Ser Ala Asn Gln Tyr Glu Val Arg Ile Asn Ser Glu Gly Arg Arg  
 740 745 750  
 Glu Leu Leu Asp His Ser Gly Glu Trp Ile Asn Lys Glu Glu Ser Ile  
 755 760 765  
 Ile Lys Asp Ile Ser Ser Lys Glu Tyr Ile Ser Phe Asn Pro Lys Glu  
 770 775 780  
 Asn Lys Ile Thr Val Lys Ser Lys Asn Leu Pro Glu Leu Ser Thr Leu  
 785 790 795 800  
 Leu Gln Glu Ile Arg Asn Asn Ser Asn Ser Ser Asp Ile Glu Leu Glu  
 805 810 815  
 Glu Lys Val Met Leu Thr Glu Cys Glu Ile Asn Val Ile Ser Asn Ile  
 820 825 830  
 Asp Thr Gln Ile Val Glu Glu Arg Ile Glu Glu Ala Lys Asn Leu Thr  
 835 840 845  
 Ser Asp Ser Ile Asn Tyr Ile Lys Asp Glu Phe Lys Leu Ile Glu Ser  
 850 855 860  
 Ile Ser Asp Ala Leu Cys Asp Leu Lys Gln Gln Asn Glu Leu Glu Asp  
 865 870 875 880  
 Ser His Phe Ile Ser Phe Glu Asp Ile Ser Glu Thr Asp Glu Gly Phe  
 885 890 895  
 Ser Ile Arg Phe Ile Asn Lys Glu Thr Gly Glu Ser Ile Phe Val Glu  
 900 905 910  
 Thr Glu Lys Thr Ile Phe Ser Glu Tyr Ala Asn His Ile Thr Glu Glu  
 915 920 925  
 Ile Ser Lys Ile Lys Gly Thr Ile Phe Asp Thr Val Asn Gly Lys Leu  
 930 935 940  
 Val Lys Lys Val Asn Leu Asp Thr Thr His Glu Val Asn Thr Leu Asn  
 945 950 955 960  
 Ala Ala Phe Phe Ile Gln Ser Leu Ile Glu Tyr Asn Ser Ser Lys Glu  
 965 970 975  
 Ser Leu Ser Asn Leu Ser Val Ala Met Lys Val Gln Val Tyr Ala Gln  
 980 985 990  
 Leu Phe Ser Thr Gly Leu Asn Thr Ile Thr Asp Ala Ala Lys Val Val  
 995 1000 1005  
 Glu Leu Val Ser Thr Ala Leu Asp Glu Thr Ile Asp Leu Leu Pro Thr  
 1010 1015 1020  
 Leu Ser Glu Gly Leu Pro Ile Ile Ala Thr Ile Ile Asp Gly Val Ser  
 1025 1030 1035 1040  
 Leu Gly Ala Ala Ile Lys Glu Leu Ser Glu Thr Ser Asp Pro Leu Leu  
 1045 1050 1055

Arg Gln Glu Ile Glu Ala Lys Ile Gly Ile Met Ala Val Asn Leu Thr  
 1060 1065 1070  
 Thr Ala Thr Thr Ala Ile Ile Thr Ser Ser Leu Gly Ile Ala Ser Gly  
 1075 1080 1085  
 Phe Ser Ile Leu Leu Val Pro Leu Ala Gly Ile Ser Ala Gly Ile Pro  
 1090 1095 1100  
 Ser Leu Val Asn Asn Glu Leu Val Leu Arg Asp Lys Ala Thr Lys Val  
 1105 1110 1115 1120  
 Val Asp Tyr Phe Lys His Val Ser Leu Val Glu Thr Glu Gly Val Phe  
 1125 1130 1135  
 Thr Leu Leu Asp Asp Lys Ile Met Met Pro Gln Asp Asp Leu Val Ile  
 1140 1145 1150  
 Ser Glu Ile Asp Phe Asn Asn Asn Ser Ile Val Leu Gly Lys Cys Glu  
 1155 1160 1165  
 Ile Trp Arg Met Glu Gly Gly Ser Gly His Thr Val Thr Asp Asp Ile  
 1170 1175 1180  
 Asp His Phe Phe Ser Ala Pro Ser Ile Thr Tyr Arg Glu Pro His Leu  
 1185 1190 1195 1200  
 Ser Ile Tyr Asp Val Leu Glu Val Gln Lys Glu Glu Leu Asp Leu Ser  
 1205 1210 1215  
 Lys Asp Leu Met Val Leu Pro Asn Ala Pro Asn Arg Val Phe Ala Trp  
 1220 1225 1230  
 Glu Thr Gly Trp Thr Pro Gly Leu Arg Ser Leu Glu Asn Asp Gly Thr  
 1235 1240 1245  
 Lys Leu Leu Asp Arg Ile Arg Asp Asn Tyr Glu Gly Glu Phe Tyr Trp  
 1250 1255 1260  
 Arg Tyr Phe Ala Phe Ile Ala Asp Ala Leu Ile Thr Thr Leu Lys Pro  
 1265 1270 1275 1280  
 Arg Tyr Glu Asp Thr Asn Ile Arg Ile Asn Leu Asp Ser Asn Thr Arg  
 1285 1290 1295  
 Ser Phe Ile Val Pro Ile Ile Thr Thr Glu Tyr Ile Arg Glu Lys Leu  
 1300 1305 1310  
 Ser Tyr Ser Phe Tyr Gly Ser Gly Gly Thr Tyr Ala Leu Ser Leu Ser  
 1315 1320 1325  
 Gln Tyr Asn Met Gly Ile Asn Ile Glu Leu Ser Glu Ser Asp Val Trp  
 1330 1335 1340  
 Ile Ile Asp Val Asp Asn Val Val Arg Asp Val Thr Ile Glu Ser Asp  
 1345 1350 1355 1360  
 Lys Ile Lys Lys Gly Asp Leu Ile Glu Gly Ile Leu Ser Thr Leu Ser  
 1365 1370 1375  
 Ile Glu Glu Asn Lys Ile Ile Leu Asn Ser His Glu Ile Asn Phe Ser  
 1380 1385 1390

Gly Glu Val Asn Gly Ser Asn Gly Phe Val Ser Leu Thr Phe Ser Ile  
 1395 1400 1405  
 Leu Glu Gly Ile Asn Ala Ile Ile Glu Val Asp Leu Leu Ser Lys Ser  
 1410 1415 1420  
 Tyr Lys Leu Leu Ile Ser Gly Glu Leu Lys Ile Leu Met Leu Asn Ser  
 1425 1430 1435 1440  
 Asn His Ile Gln Gln Lys Ile Asp Tyr Ile Gly Phe Asn Ser Glu Leu  
 1445 1450 1455  
 Gln Lys Asn Ile Pro Tyr Ser Phe Val Asp Ser Glu Gly Lys Glu Asn  
 1460 1465 1470  
 Gly Phe Ile Asn Gly Ser Thr Lys Glu Gly Leu Phe Val Ser Glu Leu  
 1475 1480 1485  
 Pro Asp Val Val Leu Ile Ser Lys Val Tyr Met Asp Asp Ser Lys Pro  
 1490 1495 1500  
 Ser Phe Gly Tyr Tyr Ser Asn Asn Leu Lys Asp Val Lys Val Ile Thr  
 1505 1510 1515 1520  
 Lys Asp Asn Val Asn Ile Leu Thr Gly Tyr Tyr Leu Lys Asp Asp Ile  
 1525 1530 1535  
 Lys Ile Ser Leu Ser Leu Thr Leu Gln Asp Glu Lys Thr Ile Lys Leu  
 1540 1545 1550  
 Asn Ser Val His Leu Asp Glu Ser Gly Val Ala Glu Ile Leu Lys Phe  
 1555 1560 1565  
 Met Asn Arg Lys Gly Asn Thr Asn Thr Ser Asp Ser Leu Met Ser Phe  
 1570 1575 1580  
 Leu Glu Ser Met Asn Ile Lys Ser Ile Phe Val Asn Phe Leu Gln Ser  
 1585 1590 1595 1600  
 Asn Ile Lys Phe Ile Leu Asp Ala Asn Phe Ile Ile Ser Gly Thr Thr  
 1605 1610 1615  
 Ser Ile Gly Gln Phe Glu Phe Ile Cys Asp Glu Asn Asp Asn Ile Gln  
 1620 1625 1630  
 Pro Tyr Phe Ile Lys Phe Asn Thr Leu Glu Thr Asn Tyr Thr Leu Tyr  
 1635 1640 1645  
 Val Gly Asn Arg Gln Asn Met Ile Val Glu Pro Asn Tyr Asp Leu Asp  
 1650 1655 1660  
 Asp Ser Gly Asp Ile Ser Ser Thr Val Ile Asn Phe Ser Gln Lys Tyr  
 1665 1670 1675 1680  
 Leu Tyr Gly Ile Asp Ser Cys Val Asn Lys Val Val Ile Ser Pro Asn  
 1685 1690 1695  
 Ile Tyr Thr Asp Glu Ile Asn Ile Thr Pro Val Tyr Glu Thr Asn Asn  
 1700 1705 1710  
 Thr Tyr Pro Glu Val Ile Val Leu Asp Ala Asn Tyr Ile Asn Glu Lys  
 1715 1720 1725

Ile Asn Val Asn Ile Asn Asp Leu Ser Ile Arg Tyr Val Trp Ser Asn  
 1730 1735 1740  
 Asp Gly Asn Asp Phe Ile Leu Met Ser Thr Ser Glu Glu Asn Lys Val  
 1745 1750 1755 1760  
 Ser Gln Val Lys Ile Arg Phe Val Asn Val Phe Lys Asp Lys Thr Leu  
 1765 1770 1775  
 Ala Asn Lys Leu Ser Phe Asn Phe Ser Asp Lys Gln Asp Val Pro Val  
 1780 1785 1790  
 Ser Glu Ile Ile Leu Ser Phe Thr Pro Ser Tyr Tyr Glu Asp Gly Leu  
 1795 1800 1805  
 Ile Gly Tyr Asp Leu Gly Leu Val Ser Leu Tyr Asn Glu Lys Phe Tyr  
 1810 1815 1820  
 Ile Asn Asn Phe Gly Met Met Val Ser Gly Leu Ile Tyr Ile Asn Asp  
 1825 1830 1835 1840  
 Ser Leu Tyr Tyr Phe Lys Pro Pro Val Asn Asn Leu Ile Thr Gly Phe  
 1845 1850 1855  
 Val Thr Val Gly Asp Asp Lys Tyr Tyr Phe Asn Pro Ile Asn Gly Gly  
 1860 1865 1870  
 Ala Ala Ser Ile Gly Glu Thr Ile Ile Asp Asp Lys Asn Tyr Tyr Phe  
 1875 1880 1885  
 Asn Gln Ser Gly Val Leu Gln Thr Gly Val Phe Ser Thr Glu Asp Gly  
 1890 1895 1900  
 Phe Lys Tyr Phe Ala Pro Ala Asn Thr Leu Asp Glu Asn Leu Glu Gly  
 1905 1910 1915 1920  
 Glu Ala Ile Asp Phe Thr Gly Lys Leu Ile Ile Asp Glu Asn Ile Tyr  
 1925 1930 1935  
 Tyr Phe Asp Asp Asn Tyr Arg Gly Ala Val Glu Trp Lys Glu Leu Asp  
 1940 1945 1950  
 Gly Glu Met His Tyr Phe Ser Pro Glu Thr Gly Lys Ala Phe Lys Gly  
 1955 1960 1965  
 Leu Asn Gln Ile Gly Asp Tyr Lys Tyr Tyr Phe Asn Ser Asp Gly Val  
 1970 1975 1980  
 Met Gln Lys Gly Phe Val Ser Ile Asn Asp Asn Lys His Tyr Phe Asp  
 1985 1990 1995 2000  
 Asp Ser Gly Val Met Lys Val Gly Tyr Thr Glu Ile Asp Gly Lys His  
 2005 2010 2015  
 Phe Tyr Phe Ala Glu Asn Gly Glu Met Gln Ile Gly Val Phe Asn Thr  
 2020 2025 2030  
 Glu Asp Gly Phe Lys Tyr Phe Ala His His Asn Glu Asp Leu Gly Asn  
 2035 2040 2045  
 Glu Glu Gly Glu Glu Ile Ser Tyr Ser Gly Ile Leu Asn Phe Asn Asn  
 2050 2055 2060

Lys Ile Tyr Tyr Phe Asp Asp Ser Phe Thr Ala Val Val Gly Trp Lys  
 2065 2070 2075 2080  
 Asp Leu Glu Asp Gly Ser Lys Tyr Tyr Phe Asp Glu Asp Thr Ala Glu  
 2085 2090 2095  
 Ala Tyr Ile Gly Leu Ser Leu Ile Asn Asp Gly Gln Tyr Tyr Phe Asn  
 2100 2105 2110  
 Asp Asp Gly Ile Met Gln Val Gly Phe Val Thr Ile Asn Asp Lys Val  
 2115 2120 2125  
 Phe Tyr Phe Ser Asp Ser Gly Ile Ile Glu Ser Gly Val Gln Asn Ile  
 2130 2135 2140  
 Asp Asp Asn Tyr Phe Tyr Ile Asp Asp Asn Gly Ile Val Gln Ile Gly  
 2145 2150 2155 2160  
 Val Phe Asp Thr Ser Asp Gly Tyr Lys Tyr Phe Ala Pro Ala Asn Thr  
 2165 2170 2175  
 Val Asn Asp Asn Ile Tyr Gly Gln Ala Val Glu Tyr Ser Gly Leu Val  
 2180 2185 2190  
 Arg Val Gly Glu Asp Val Tyr Tyr Phe Gly Glu Thr Tyr Thr Ile Glu  
 2195 2200 2205  
 Thr Gly Trp Ile Tyr Asp Met Glu Asn Glu Ser Asp Lys Tyr Tyr Phe  
 2210 2215 2220  
 Asn Pro Glu Thr Lys Lys Ala Cys Lys Gly Ile Asn Leu Ile Asp Asp  
 2225 2230 2235 2240  
 Ile Lys Tyr Tyr Phe Asp Glu Lys Gly Ile Met Arg Thr Gly Leu Ile  
 2245 2250 2255  
 Ser Phe Glu Asn Asn Asn Tyr Tyr Phe Asn Glu Asn Gly Glu Met Gln  
 2260 2265 2270  
 Phe Gly Tyr Ile Asn Ile Glu Asp Lys Met Phe Tyr Phe Gly Glu Asp  
 2275 2280 2285  
 Gly Val Met Gln Ile Gly Val Phe Asn Thr Pro Asp Gly Phe Lys Tyr  
 2290 2295 2300  
 Phe Ala His Gln Asn Thr Leu Asp Glu Asn Phe Glu Gly Glu Ser Ile  
 2305 2310 2315 2320  
 Asn Tyr Thr Gly Trp Leu Asp Leu Asp Glu Lys Arg Tyr Tyr Phe Thr  
 2325 2330 2335  
 Asp Glu Tyr Ile Ala Ala Thr Gly Ser Val Ile Ile Asp Gly Glu Glu  
 2340 2345 2350  
 Tyr Tyr Phe Asp Pro Asp Thr Ala Gln Leu Val Ile Ser Glu  
 2355 2360 2365

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TAGAAAAAAT GGCAAATGT

19

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTTCATCTTG TAGAGTCAAA G

21

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATGCCACAA GATGATTTAG TG

22

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTAATTGAGC TGTATCAGGA TC

22

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGGAATTCCT AGAAAAAATG GCAAATG

27

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCTCTAGAAT GACCATAAGC TAGCCA

26

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGGAATTCGA GTTGGTAGAA AGGTGGA

27

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGGAATTCGG TTATTATCTT AAGGATG

27

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 28 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGGAATTCTT GATAACTGGA TTTGTGAC

28

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 511 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: unknown  
    (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu	Ile	Thr	Gly	Phe	Val	Thr	Val	Gly	Asp	Asp	Lys	Tyr	Tyr	Phe	Asn	
1				5				10						15		
Pro	Ile	Asn	Gly	Gly	Ala	Ala	Ser	Ile	Gly	Glu	Thr	Ile	Ile	Asp	Asp	
		20					25						30			
Lys	Asn	Tyr	Tyr	Phe	Asn	Gln	Ser	Gly	Val	Leu	Gln	Thr	Gly	Val	Phe	
		35				40						45				
Ser	Thr	Glu	Asp	Gly	Phe	Lys	Tyr	Phe	Ala	Pro	Ala	Asn	Thr	Leu	Asp	
	50				55					60						
Glu	Asn	Leu	Glu	Gly	Glu	Ala	Ile	Asp	Phe	Thr	Gly	Lys	Leu	Ile	Ile	
65				70					75					80		
Asp	Glu	Asn	Ile	Tyr	Phe	Asp	Asp	Asn	Tyr	Arg	Gly	Ala	Val	Glu		
			85				90						95			
Trp	Lys	Glu	Leu	Asp	Gly	Glu	Met	His	Tyr	Phe	Ser	Pro	Glu	Thr	Gly	
		100					105						110			
Lys	Ala	Phe	Lys	Gly	Leu	Asn	Gln	Ile	Gly	Asp	Tyr	Lys	Tyr	Tyr	Phe	
		115				120						125				
Asn	Ser	Asp	Gly	Val	Met	Gln	Lys	Gly	Phe	Val	Ser	Ile	Asn	Asp	Asn	
	130					135					140					
Lys	His	Tyr	Phe	Asp	Asp	Ser	Gly	Val	Met	Lys	Val	Gly	Tyr	Thr	Glu	
145				150					155					160		
Ile	Asp	Gly	Lys	His	Phe	Tyr	Phe	Ala	Glu	Asn	Gly	Glu	Met	Gln	Ile	
			165				170						175			
Gly	Val	Phe	Asn	Thr	Glu	Asp	Gly	Phe	Lys	Tyr	Phe	Ala	His	His	Asn	
		180					185						190			



Glu Asp Leu Gly Asn Glu Glu Gly Glu Glu Ile Ser Tyr Ser Gly Ile  
 195 200 205  
 Leu Asn Phe Asn Asn Lys Ile Tyr Tyr Phe Asp Asp Ser Phe Thr Ala  
 210 215 220  
 Val Val Gly Trp Lys Asp Leu Glu Asp Gly Ser Lys Tyr Tyr Phe Asp  
 225 230 235 240  
 Glu Asp Thr Ala Glu Ala Tyr Ile Gly Leu Ser Leu Ile Asn Asp Gly  
 245 250 255  
 Gln Tyr Tyr Phe Asn Asp Asp Gly Ile Met Gln Val Gly Phe Val Thr  
 260 265 270  
 Ile Asn Asp Lys Val Phe Tyr Phe Ser Asp Ser Gly Ile Ile Glu Ser  
 275 280 285  
 Gly Val Gln Asn Ile Asp Asp Asn Tyr Phe Tyr Ile Asp Asp Asn Gly  
 290 295 300  
 Ile Val Gln Ile Gly Val Phe Asp Thr Ser Asp Gly Tyr Lys Tyr Phe  
 305 310 315 320  
 Ala Pro Ala Asn Thr Val Asn Asp Asn Ile Tyr Gly Gln Ala Val Glu  
 325 330 335  
 Tyr Ser Gly Leu Val Arg Val Gly Glu Asp Val Tyr Tyr Phe Gly Glu  
 340 345 350  
 Thr Tyr Thr Ile Glu Thr Gly Trp Ile Tyr Asp Met Glu Asn Glu Ser  
 355 360 365  
 Asp Lys Tyr Tyr Phe Asn Pro Glu Thr Lys Lys Ala Cys Lys Gly Ile  
 370 375 380  
 Asn Leu Ile Asp Asp Ile Lys Tyr Tyr Phe Asp Glu Lys Gly Ile Met  
 385 390 395 400  
 Arg Thr Gly Leu Ile Ser Phe Glu Asn Asn Asn Tyr Tyr Phe Asn Glu  
 405 410 415  
 Asn Gly Glu Met Gln Phe Gly Tyr Ile Asn Ile Glu Asp Lys Met Phe  
 420 425 430  
 Tyr Phe Gly Glu Asp Gly Val Met Gln Ile Gly Val Phe Asn Thr Pro  
 435 440 445  
 Asp Gly Phe Lys Tyr Phe Ala His Gln Asn Thr Leu Asp Glu Asn Phe  
 450 455 460  
 Glu Gly Glu Ser Ile Asn Tyr Thr Gly Trp Leu Asp Leu Asp Glu Lys  
 465 470 475 480  
 Arg Tyr Tyr Phe Thr Asp Glu Tyr Ile Ala Ala Thr Gly Ser Val Ile  
 485 490 495  
 Ile Asp Gly Glu Glu Tyr Tyr Phe Asp Pro Asp Thr Ala Gln Leu  
 500 505 510

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 608 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ser Glu Glu Asn Lys Val Ser Gln Val Lys Ile Arg Phe Val Asn Val  
1 5 10 15  
Phe Lys Asp Lys Thr Leu Ala Asn Lys Leu Ser Phe Asn Phe Ser Asp  
20 25 30  
Lys Gln Asp Val Pro Val Ser Glu Ile Ile Leu Ser Phe Thr Pro Ser  
35 40 45  
Tyr Tyr Glu Asp Gly Leu Ile Gly Tyr Asp Leu Gly Leu Val Ser Leu  
50 55 60  
Tyr Asn Glu Lys Phe Tyr Ile Asn Asn Phe Gly Met Met Val Ser Gly  
65 70 75 80  
Leu Ile Tyr Ile Asn Asp Ser Leu Tyr Tyr Phe Lys Pro Pro Val Asn  
85 90 95  
Asn Leu Ile Thr Gly Phe Val Thr Val Gly Asp Asp Lys Tyr Tyr Phe  
100 105 110  
Asn Pro Ile Asn Gly Gly Ala Ala Ser Ile Gly Glu Thr Ile Ile Asp  
115 120 125  
Asp Lys Asn Tyr Tyr Phe Asn Gln Ser Gly Val Leu Gln Thr Gly Val  
130 135 140  
Phe Ser Thr Glu Asp Gly Phe Lys Tyr Phe Ala Pro Ala Asn Thr Leu  
145 150 155 160  
Asp Glu Asn Leu Glu Gly Glu Ala Ile Asp Phe Thr Gly Lys Leu Ile  
165 170 175  
Ile Asp Glu Asn Ile Tyr Tyr Phe Asp Asp Asn Tyr Arg Gly Ala Val  
180 185 190  
Glu Trp Lys Glu Leu Asp Gly Glu Met His Tyr Phe Ser Pro Glu Thr  
195 200 205  
Gly Lys Ala Phe Lys Gly Leu Asn Gln Ile Gly Asp Tyr Lys Tyr Tyr  
210 215 220  
Phe Asn Ser Asp Gly Val Met Gln Lys Gly Phe Val Ser Ile Asn Asp  
225 230 235 240  
Asn Lys His Tyr Phe Asp Asp Ser Gly Val Met Lys Val Gly Tyr Thr  
245 250 255  
Glu Ile Asp Gly Lys His Phe Tyr Phe Ala Glu Asn Gly Glu Met Gln  
260 265 270

Ile Gly Val Phe Asn Thr Glu Asp Gly Phe Lys Tyr Phe Ala His His  
 275 280 285  
 Asn Glu Asp Leu Gly Asn Glu Glu Gly Glu Glu Ile Ser Tyr Ser Gly  
 290 295 300  
 Ile Leu Asn Phe Asn Asn Lys Ile Tyr Tyr Phe Asp Asp Ser Phe Thr  
 305 310 315 320  
 Ala Val Val Gly Trp Lys Asp Leu Glu Asp Gly Ser Lys Tyr Tyr Phe  
 325 330 335  
 Asp Glu Asp Thr Ala Glu Ala Tyr Ile Gly Leu Ser Leu Ile Asn Asp  
 340 345 350  
 Gly Gln Tyr Tyr Phe Asn Asp Asp Gly Ile Met Gln Val Gly Phe Val  
 355 360 365  
 Thr Ile Asn Asp Lys Val Phe Tyr Phe Ser Asp Ser Gly Ile Ile Glu  
 370 375 380  
 Ser Gly Val Gln Asn Ile Asp Asp Asn Tyr Phe Tyr Ile Asp Asp Asn  
 385 390 395 400  
 Gly Ile Val Gln Ile Gly Val Phe Asp Thr Ser Asp Gly Tyr Lys Tyr  
 405 410 415  
 Phe Ala Pro Ala Asn Thr Val Asn Asp Asn Ile Tyr Gly Gln Ala Val  
 420 425 430  
 Glu Tyr Ser Gly Leu Val Arg Val Gly Glu Asp Val Tyr Tyr Phe Gly  
 435 440 445  
 Glu Thr Tyr Thr Ile Glu Thr Gly Trp Ile Tyr Asp Met Glu Asn Glu  
 450 455 460  
 Ser Asp Lys Tyr Tyr Phe Asn Pro Glu Thr Lys Lys Ala Cys Lys Gly  
 465 470 475 480  
 Ile Asn Leu Ile Asp Asp Ile Lys Tyr Tyr Phe Asp Glu Lys Gly Ile  
 485 490 495  
 Met Arg Thr Gly Leu Ile Ser Phe Glu Asn Asn Asn Tyr Tyr Phe Asn  
 500 505 510  
 Glu Asn Gly Glu Met Gln Phe Gly Tyr Ile Asn Ile Glu Asp Lys Met  
 515 520 525  
 Phe Tyr Phe Gly Glu Asp Gly Val Met Gln Ile Gly Val Phe Asn Thr  
 530 535 540  
 Pro Asp Gly Phe Lys Tyr Phe Ala His Gln Asn Thr Leu Asp Glu Asn  
 545 550 555 560  
 Phe Glu Gly Glu Ser Ile Asn Tyr Thr Gly Trp Leu Asp Leu Asp Glu  
 565 570 575  
 Lys Arg Tyr Tyr Phe Thr Asp Glu Tyr Ile Ala Ala Thr Gly Ser Val  
 580 585 590  
 Ile Ile Asp Gly Glu Glu Tyr Tyr Phe Asp Pro Asp Thr Ala Gln Leu  
 595 600 605

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1330 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1314

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATG GCT CGT CTG CTG TCT ACC TTC ACT GAA TAC ATC AAG AAC ATC ATC	48
Met Ala Arg Leu Leu Ser Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile	
1 5 10 15	
AAT ACC TCC ATC CTG AAC CTG CGC TAC GAA TCC AAT CAC CTG ATC GAC	96
Asn Thr Ser Ile Leu Asn Leu Arg Tyr Glu Ser Asn His Leu Ile Asp	
20 25 30	
CTG TCT CGC TAC GCT TCC AAA ATC AAC ATC GGT TCT AAA GTT AAC TTC	144
Leu Ser Arg Tyr Ala Ser Lys Ile Asn Ile Gly Ser Lys Val Asn Phe	
35 40 45	
GAT CCG ATC GAC AAG AAT CAG ATC CAG CTG TTC AAT CTG GAA TCT TCC	192
Asp Pro Ile Asp Lys Asn Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser	
50 55 60	
AAA ATC GAA GTT ATC CTG AAG AAT GCT ATC GTA TAC AAC TCT ATG TAC	240
Lys Ile Glu Val Ile Leu Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr	
65 70 75 80	
GAA AAC TTC TCC ACC TCC TTC TGG ATC CGT ATC CCG AAA TAC TTC AAC	288
Glu Asn Phe Ser Thr Ser Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn	
85 90 95	
TCC ATC TCT CTG AAC AAT GAA TAC ACC ATC ATC AAC TGC ATG GAA AAC	336
Ser Ile Ser Leu Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn	
100 105 110	
AAT TCT GGT TGG AAA GTA TCT CTG AAC TAC GGT GAA ATC ATC TGG ACT	384
Asn Ser Gly Trp Lys Val Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr	
115 120 125	
CTG CAG GAC ACT CAG GAA ATC AAA CAG CGT GTT GTA TTC AAA TAC TCT	432
Leu Gln Asp Thr Gln Glu Ile Lys Gln Arg Val Val Phe Lys Tyr Ser	
130 135 140	
CAG ATG ATC AAC ATC TCT GAC TAC ATC AAT CGC TGG ATC TTC GTT ACC	480
Gln Met Ile Asn Ile Ser Asp Tyr Ile Asn Arg Trp Ile Phe Val Thr	
145 150 155 160	
ATC ACC AAC AAT CGT CTG AAT AAC TCC AAA ATC TAC ATC AAC GGC CGT	528
Ile Thr Asn Asn Arg Leu Asn Asn Ser Lys Ile Tyr Ile Asn Gly Arg	
165 170 175	

CTG ATC GAC CAG AAA CCG ATC TCC AAT CTG GGT AAC ATC CAC GCT TCT Leu Ile Asp Gln Lys Pro Ile Ser Asn Leu Gly Asn Ile His Ala Ser 180 185 190	576
AAT AAC ATC ATG TTC AAA CTG GAC GGT TGT CGT GAC ACT CAC CGC TAC Asn Asn Ile Met Phe Lys Leu Asp Gly Cys Arg Asp Thr His Arg Tyr 195 200 205	624
ATC TGG ATC AAA TAC TTC AAT CTG TTC GAC AAA GAA CTG AAC GAA AAA Ile Trp Ile Lys Tyr Phe Asn Leu Phe Asp Lys Glu Leu Asn Glu Lys 210 215 220	672
GAA ATC AAA GAC CTG TAC GAC AAC CAG TCC AAT TCT GGT ATC CTG AAA Glu Ile Lys Asp Leu Tyr Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys 225 230 235 240	720
GAC TTC TGG GGT GAC TAC CTG CAG TAC GAC AAA CCG TAC TAC ATG CTG Asp Phe Trp Gly Asp Tyr Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu 245 250 255	768
AAT CTG TAC GAT CCG AAC AAA TAC GTT GAC GTC AAC AAT GTA GGT ATC Asn Leu Tyr Asp Pro Asn Lys Tyr Val Asp Val Asn Asn Val Gly Ile 260 265 270	816
CGC GGT TAC ATG TAC CTG AAA GGT CCG CGT GGT TCT GTT ATG ACT ACC Arg Gly Tyr Met Tyr Leu Lys Gly Pro Arg Gly Ser Val Met Thr Thr 275 280 285	864
AAC ATC TAC CTG AAC TCT TCC CTG TAC CGT GGT ACC AAA TTC ATC ATC Asn Ile Tyr Leu Asn Ser Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile 290 295 300	912
AAG AAA TAC GCG TCT GGT AAC AAG GAC AAT ATC GTT CGC AAC AAT GAT Lys Lys Tyr Ala Ser Gly Asn Lys Asp Asn Ile Val Arg Asn Asn Asp 305 310 315 320	960
CGT GTA TAC ATC AAT GTT GTA GTT AAG AAC AAA GAA TAC CGT CTG GCT Arg Val Tyr Ile Asn Val Val Val Lys Asn Lys Glu Tyr Arg Leu Ala 325 330 335	1008
ACC AAT GCT TCT CAG GCT GGT GTA GAA AAG ATC TTG TCT GCT CTG GAA Thr Asn Ala Ser Gln Ala Gly Val Glu Lys Ile Leu Ser Ala Leu Glu 340 345 350	1056
ATC CCG GAC GTT GGT AAT CTG TCT CAG GTA GTT GTA ATG AAA TCC AAG Ile Pro Asp Val Gly Asn Leu Ser Gln Val Val Val Met Lys Ser Lys 355 360 365	1104
AAC GAC CAG GGT ATC ACT AAC AAA TGC AAA ATG AAT CTG CAG GAC AAC Asn Asp Gln Gly Ile Thr Asn Lys Cys Lys Met Asn Leu Gln Asp Asn 370 375 380	1152
AAT GGT AAC GAT ATC GGT TTC ATC GGT TTC CAC CAG TTC AAC AAT ATC Asn Gly Asn Asp Ile Gly Phe Ile Gly Phe His Gln Phe Asn Asn Ile 385 390 395 400	1200
GCT AAA CTG GTT GCT TCC AAC TGG TAC AAT CGT CAG ATC GAA CGT TCC Ala Lys Leu Val Ala Ser Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser 405 410 415	1248

TCT CGC ACT CTG GGT TGC TCT TGG GAG TTC ATC CCG GTT GAT GAC GGT 1296  
Ser Arg Thr Leu Gly Cys Ser Trp Glu Phe Ile Pro Val Asp Asp Gly  
420 425 430

TGG GGT GAA CGT CCG CTG TAACCCGGA AAGCTT 1330  
Trp Gly Glu Arg Pro Leu  
435

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 438 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met 1	Ala	Arg	Leu	Leu 5	Ser	Thr	Phe	Thr	Glu 10	Tyr	Ile	Lys	Asn	Ile 15	Ile
Asn	Thr	Ser	Ile 20	Leu	Asn	Leu	Arg	Tyr 25	Glu	Ser	Asn	His	Leu 30	Ile	Asp
Leu	Ser	Arg 35	Tyr	Ala	Ser	Lys	Ile 40	Asn	Ile	Gly	Ser	Lys 45	Val	Asn	Phe
Asp	Pro 50	Ile	Asp	Lys	Asn	Gln 55	Ile	Gln	Leu	Phe	Asn 60	Leu	Glu	Ser	Ser
Lys 65	Ile	Glu	Val	Ile 70	Leu	Lys	Asn	Ala	Ile	Val 75	Tyr	Asn	Ser	Met	Tyr 80
Glu	Asn	Phe	Ser	Thr 85	Ser	Phe	Trp	Ile	Arg 90	Ile	Pro	Lys	Tyr	Phe 95	Asn
Ser	Ile	Ser	Leu 100	Asn	Asn	Glu	Tyr	Thr 105	Ile	Ile	Asn	Cys	Met 110	Glu	Asn
Asn	Ser	Gly 115	Trp	Lys	Val	Ser	Leu 120	Asn	Tyr	Gly	Glu	Ile 125	Ile	Trp	Thr
Leu	Gln 130	Asp	Thr	Gln	Glu	Ile 135	Lys	Gln	Arg	Val	Val 140	Phe	Lys	Tyr	Ser
Gln 145	Met	Ile	Asn	Ile 150	Ser	Asp	Tyr	Ile	Asn	Arg 155	Trp	Ile	Phe	Val	Thr 160
Ile	Thr	Asn	Asn	Arg 165	Leu	Asn	Asn	Ser	Lys 170	Ile	Tyr	Ile	Asn	Gly 175	Arg
Leu	Ile	Asp	Gln 180	Lys	Pro	Ile	Ser	Asn 185	Leu	Gly	Asn	Ile	His 190	Ala	Ser
Asn	Asn	Ile 195	Met	Phe	Lys	Leu	Asp 200	Gly	Cys	Arg	Asp	Thr 205	His	Arg	Tyr
Ile	Trp 210	Ile	Lys	Tyr	Phe 215	Asn	Leu	Phe	Asp	Lys 220	Glu	Leu	Asn	Glu	Lys

Glu Ile Lys Asp Leu Tyr Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys  
 225 230 235 240  
 Asp Phe Trp Gly Asp Tyr Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu  
 245 250 255  
 Asn Leu Tyr Asp Pro Asn Lys Tyr Val Asp Val Asn Asn Val Gly Ile  
 260 265 270  
 Arg Gly Tyr Met Tyr Leu Lys Gly Pro Arg Gly Ser Val Met Thr Thr  
 275 280 285  
 Asn Ile Tyr Leu Asn Ser Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile  
 290 295 300  
 Lys Lys Tyr Ala Ser Gly Asn Lys Asp Asn Ile Val Arg Asn Asn Asp  
 305 310 315 320  
 Arg Val Tyr Ile Asn Val Val Val Lys Asn Lys Glu Tyr Arg Leu Ala  
 325 330 335  
 Thr Asn Ala Ser Gln Ala Gly Val Glu Lys Ile Leu Ser Ala Leu Glu  
 340 345 350  
 Ile Pro Asp Val Gly Asn Leu Ser Gln Val Val Val Met Lys Ser Lys  
 355 360 365  
 Asn Asp Gln Gly Ile Thr Asn Lys Cys Lys Met Asn Leu Gln Asp Asn  
 370 375 380  
 Asn Gly Asn Asp Ile Gly Phe Ile Gly Phe His Gln Phe Asn Asn Ile  
 385 390 395 400  
 Ala Lys Leu Val Ala Ser Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser  
 405 410 415  
 Ser Arg Thr Leu Gly Cys Ser Trp Glu Phe Ile Pro Val Asp Asp Gly  
 420 425 430  
 Trp Gly Glu Arg Pro Leu  
 435

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 23 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Met Gly His His His His His His His His His His Ser Ser Gly His  
 1 5 10 15  
 Ile Glu Gly Arg His Met Ala  
 20

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1402 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1386

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATG GGC CAT CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT	48
Met Gly His His His His His His His His His His Ser Ser Gly His	
1 5 10 15	
ATC GAA GGT CGT CAT ATG GCT AGC ATG GCT CGT CTG CTG TCT ACC TTC	96
Ile Glu Gly Arg His Met Ala Ser Met Ala Arg Leu Leu Ser Thr Phe	
20 25 30	
ACT GAA TAC ATC AAG AAC ATC ATC AAT ACC TCC ATC CTG AAC CTG CGC	144
Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg	
35 40 45	
TAC GAA TCC AAT CAC CTG ATC GAC CTG TCT CGC TAC GCT TCC AAA ATC	192
Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile	
50 55 60	
AAC ATC GGT TCT AAA GTT AAC TTC GAT CCG ATC GAC AAG AAT CAG ATC	240
Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile	
65 70 75 80	
CAG CTG TTC AAT CTG GAA TCT TCC AAA ATC GAA GTT ATC CTG AAG AAT	288
Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn	
85 90 95	
GCT ATC GTA TAC AAC TCT ATG TAC GAA AAC TTC TCC ACC TCC TTC TGG	336
Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp	
100 105 110	
ATC CGT ATC CCG AAA TAC TTC AAC TCC ATC TCT CTG AAC AAT GAA TAC	384
Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr	
115 120 125	
ACC ATC ATC AAC TGC ATG GAA AAC AAT TCT GGT TGG AAA GTA TCT CTG	432
Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu	
130 135 140	
AAC TAC GGT GAA ATC ATC TGG ACT CTG CAG GAC ACT CAG GAA ATC AAA	480
Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys	
145 150 155 160	
CAG CGT GTT GTA TTC AAA TAC TCT CAG ATG ATC AAC ATC TCT GAC TAC	528
Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr	
165 170 175	



ATC AAT CGC TGG ATC TTC GTT ACC ATC ACC AAC AAT CGT CTG AAT AAC Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn 180 185 190	576
TCC AAA ATC TAC ATC AAC GGC CGT CTG ATC GAC CAG AAA CCG ATC TCC Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser 195 200 205	624
AAT CTG GGT AAC ATC CAC GCT TCT AAT AAC ATC ATG TTC AAA CTG GAC Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp 210 215 220	672
GGT TGT CGT GAC ACT CAC CGC TAC ATC TGG ATC AAA TAC TTC AAT CTG Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu 225 230 235 240	720
TTC GAC AAA GAA CTG AAC GAA AAA GAA ATC AAA GAC CTG TAC GAC AAC Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn 245 250 255	768
CAG TCC AAT TCT GGT ATC CTG AAA GAC TTC TGG GGT GAC TAC CTG CAG Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln 260 265 270	816
TAC GAC AAA CCG TAC TAC ATG CTG AAT CTG TAC GAT CCG AAC AAA TAC Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr 275 280 285	864
GTT GAC GTC AAC AAT GTA GGT ATC CGC GGT TAC ATG TAC CTG AAA GGT Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly 290 295 300	912
CCG CGT GGT TCT GTT ATG ACT ACC AAC ATC TAC CTG AAC TCT TCC CTG Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu 305 310 315 320	960
TAC CGT GGT ACC AAA TTC ATC ATC AAG AAA TAC GCG TCT GGT AAC AAG Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys 325 330 335	1008
GAC AAT ATC GTT CGC AAC AAT GAT CGT GTA TAC ATC AAT GTT GTA GTT Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val 340 345 350	1056
AAG AAC AAA GAA TAC CGT CTG GCT ACC AAT GCT TCT CAG GCT GGT GTA Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val 355 360 365	1104
GAA AAG ATC TTG TCT GCT CTG GAA ATC CCG GAC GTT GGT AAT CTG TCT Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser 370 375 380	1152
CAG GTA GTT GTA ATG AAA TCC AAG AAC GAC CAG GGT ATC ACT AAC AAA Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys 385 390 395 400	1200
TGC AAA ATG AAT CTG CAG GAC AAC AAT GGT AAC GAT ATC GGT TTC ATC Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile 405 410 415	1248

GGT TTC CAC CAG TTC AAC AAT ATC GCT AAA CTG GTT GCT TCC AAC TGG	1296
Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp	
420 425 430	
TAC AAT CGT CAG ATC GAA CGT TCC TCT CGC ACT CTG GGT TGC TCT TGG	1344
Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp	
435 440 445	
GAG TTC ATC CCG GTT GAT GAC GGT TGG GGT GAA CGT CCG CTG	1386
Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu	
450 455 460	
TAACCCGGGA AAGCTT	1402

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 462 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Met	Gly	His	His	His	His	His	His	His	His	His	His	Ser	Ser	Gly	His
1				5						10				15	
Ile	Glu	Gly	Arg	His	Met	Ala	Ser	Met	Ala	Arg	Leu	Leu	Ser	Thr	Phe
			20					25					30		
Thr	Glu	Tyr	Ile	Lys	Asn	Ile	Ile	Asn	Thr	Ser	Ile	Leu	Asn	Leu	Arg
		35					40					45			
Tyr	Glu	Ser	Asn	His	Leu	Ile	Asp	Leu	Ser	Arg	Tyr	Ala	Ser	Lys	Ile
	50					55				60					
Asn	Ile	Gly	Ser	Lys	Val	Asn	Phe	Asp	Pro	Ile	Asp	Lys	Asn	Gln	Ile
65					70				75					80	
Gln	Leu	Phe	Asn	Leu	Glu	Ser	Ser	Lys	Ile	Glu	Val	Ile	Leu	Lys	Asn
			85						90					95	
Ala	Ile	Val	Tyr	Asn	Ser	Met	Tyr	Glu	Asn	Phe	Ser	Thr	Ser	Phe	Trp
		100						105					110		
Ile	Arg	Ile	Pro	Lys	Tyr	Phe	Asn	Ser	Ile	Ser	Leu	Asn	Asn	Glu	Tyr
	115						120					125			
Thr	Ile	Ile	Asn	Cys	Met	Glu	Asn	Asn	Ser	Gly	Trp	Lys	Val	Ser	Leu
	130					135					140				
Asn	Tyr	Gly	Glu	Ile	Ile	Trp	Thr	Leu	Gln	Asp	Thr	Gln	Glu	Ile	Lys
145				150					155					160	
Gln	Arg	Val	Val	Phe	Lys	Tyr	Ser	Gln	Met	Ile	Asn	Ile	Ser	Asp	Tyr
			165						170					175	
Ile	Asn	Arg	Trp	Ile	Phe	Val	Thr	Ile	Thr	Asn	Asn	Arg	Leu	Asn	Asn
		180						185					190		

Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser  
 195 200 205  
 Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp  
 210 215 220  
 Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu  
 225 230 235 240  
 Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn  
 245 250 255  
 Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln  
 260 265 270  
 Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr  
 275 280 285  
 Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly  
 290 295 300  
 Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu  
 305 310 315 320  
 Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys  
 325 330 335  
 Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val  
 340 345 350  
 Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val  
 355 360 365  
 Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser  
 370 375 380  
 Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys  
 385 390 395 400  
 Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile  
 405 410 415  
 Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp  
 420 425 430  
 Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp  
 435 440 445  
 Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu  
 450 455 460

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3891 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..3888

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATG CAA TTT GTT AAT AAA CAA TTT AAT TAT AAA GAT CCT GTA AAT GGT	48
Met Gln Phe Val Asn Lys Gln Phe Asn Tyr Lys Asp Pro Val Asn Gly	
1 5 10 15	
GTT GAT ATT GCT TAT ATA AAA ATT CCA AAT GTA GGA CAA ATG CAA CCA	96
Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Val Gly Gln Met Gln Pro	
20 25 30	
GTA AAA GCT TTT AAA ATT CAT AAT AAA ATA TGG GTT ATT CCA GAA AGA	144
Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val Ile Pro Glu Arg	
35 40 45	
GAT ACA TTT ACA AAT CCT GAA GAA GGA GAT TTA AAT CCA CCA CCA GAA	192
Asp Thr Phe Thr Asn Pro Glu Glu Gly Asp Leu Asn Pro Pro Pro Glu	
50 55 60	
GCA AAA CAA GTT CCA GTT TCA TAT TAT GAT TCA ACA TAT TTA AGT ACA	240
Ala Lys Gln Val Pro Val Ser Tyr Tyr Asp Ser Thr Tyr Leu Ser Thr	
65 70 75 80	
GAT AAT GAA AAA GAT AAT TAT TTA AAG GGA GTT ACA AAA TTA TTT GAG	288
Asp Asn Glu Lys Asp Asn Tyr Leu Lys Gly Val Thr Lys Leu Phe Glu	
85 90 95	
AGA ATT TAT TCA ACT GAT CTT GGA AGA ATG TTG TTA ACA TCA ATA GTA	336
Arg Ile Tyr Ser Thr Asp Leu Gly Arg Met Leu Leu Thr Ser Ile Val	
100 105 110	
AGG GGA ATA CCA TTT TGG GGT GGA AGT ACA ATA GAT ACA GAA TTA AAA	384
Arg Gly Ile Pro Phe Trp Gly Gly Ser Thr Ile Asp Thr Glu Leu Lys	
115 120 125	
GTT ATT GAT ACT AAT TGT ATT AAT GTG ATA CAA CCA GAT GGT AGT TAT	432
Val Ile Asp Thr Asn Cys Ile Asn Val Ile Gln Pro Asp Gly Ser Tyr	
130 135 140	
AGA TCA GAA GAA CTT AAT CTA GTA ATA ATA GGA CCC TCA GCT GAT ATT	480
Arg Ser Glu Glu Leu Asn Leu Val Ile Ile Gly Pro Ser Ala Asp Ile	
145 150 155 160	
ATA CAG TTT GAA TGT AAA AGC TTT GGA CAT GAA GTT TTG AAT CTT ACG	528
Ile Gln Phe Glu Cys Lys Ser Phe Gly His Glu Val Leu Asn Leu Thr	
165 170 175	
CGA AAT GGT TAT GGC TCT ACT CAA TAC ATT AGA TTT AGC CCA GAT TTT	576
Arg Asn Gly Tyr Gly Ser Thr Gln Tyr Ile Arg Phe Ser Pro Asp Phe	
180 185 190	
ACA TTT GGT TTT GAG GAG TCA CTT GAA GTT GAT ACA AAT CCT CTT TTA	624
Thr Phe Gly Phe Glu Glu Ser Leu Glu Val Asp Thr Asn Pro Leu Leu	
195 200 205	
GGT GCA GGC AAA TTT GCT ACA GAT CCA GCA GTA ACA TTA GCA CAT GAA	672
Gly Ala Gly Lys Phe Ala Thr Asp Pro Ala Val Thr Leu Ala His Glu	
210 215 220	

CTT ATA CAT GCT GGA CAT AGA TTA TAT GGA ATA GCA ATT AAT CCA AAT Leu Ile His Ala Gly His Arg Leu Tyr Gly Ile Ala Ile Asn Pro Asn 225 230 235 240	720
AGG GTT TTT AAA GTA AAT ACT AAT GCC TAT TAT GAA ATG AGT GGG TTA Arg Val Phe Lys Val Asn Thr Asn Ala Tyr Tyr Glu Met Ser Gly Leu 245 250 255	768
GAA GTA AGC TTT GAG GAA CTT AGA ACA TTT GGG GGA CAT GAT GCA AAG Glu Val Ser Phe Glu Glu Leu Arg Thr Phe Gly Gly His Asp Ala Lys 260 265 270	816
TTT ATA GAT AGT TTA CAG GAA AAC GAA TTT CGT CTA TAT TAT TAT AAT Phe Ile Asp Ser Leu Gln Glu Asn Glu Phe Arg Leu Tyr Tyr Tyr Asn 275 280 285	864
AAG TTT AAA GAT ATA GCA AGT ACA CTT AAT AAA GCT AAA TCA ATA GTA Lys Phe Lys Asp Ile Ala Ser Thr Leu Asn Lys Ala Lys Ser Ile Val 290 295 300	912
GGT ACT ACT GCT TCA TTA CAG TAT ATG AAA AAT GTT TTT AAA GAG AAA Gly Thr Thr Ala Ser Leu Gln Tyr Met Lys Asn Val Phe Lys Glu Lys 305 310 315 320	960
TAT CTC CTA TCT GAA GAT ACA TCT GGA AAA TTT TCG GTA GAT AAA TTA Tyr Leu Leu Ser Glu Asp Thr Ser Gly Lys Phe Ser Val Asp Lys Leu 325 330 335	1008
AAA TTT GAT AAG TTA TAC AAA ATG TTA ACA GAG ATT TAC ACA GAG GAT Lys Phe Asp Lys Leu Tyr Lys Met Leu Thr Glu Ile Tyr Thr Glu Asp 340 345 350	1056
AAT TTT GTT AAG TTT TTT AAA GTA CTT AAC AGA AAA ACA TAT TTG AAT Asn Phe Val Lys Phe Phe Lys Val Leu Asn Arg Lys Thr Tyr Leu Asn 355 360 365	1104
TTT GAT AAA GCC GTA TTT AAG ATA AAT ATA GTA CCT AAG GTA AAT TAC Phe Asp Lys Ala Val Phe Lys Ile Asn Ile Val Pro Lys Val Asn Tyr 370 375 380	1152
ACA ATA TAT GAT GGA TTT AAT TTA AGA AAT ACA AAT TTA GCA GCA AAC Thr Ile Tyr Asp Gly Phe Asn Leu Arg Asn Thr Asn Leu Ala Ala Asn 385 390 395 400	1200
TTT AAT GGT CAA AAT ACA GAA ATT AAT AAT ATG AAT TTT ACT AAA CTA Phe Asn Gly Gln Asn Thr Glu Ile Asn Asn Met Asn Phe Thr Lys Leu 405 410 415	1248
AAA AAT TTT ACT GGA TTG TTT GAA TTT TAT AAG TTG CTA TGT GTA AGA Lys Asn Phe Thr Gly Leu Phe Glu Phe Tyr Lys Leu Leu Cys Val Arg 420 425 430	1296
GGG ATA ATA ACT TCT AAA ACT AAA TCA TTA GAT AAA GGA TAC AAT AAG Gly Ile Ile Thr Ser Lys Thr Lys Ser Leu Asp Lys Gly Tyr Asn Lys 435 440 445	1344
GCA TTA AAT GAT TTA TGT ATC AAA GTT AAT AAT TGG GAC TTG TTT TTT Ala Leu Asn Asp Leu Cys Ile Lys Val Asn Asn Trp Asp Leu Phe Phe 450 455 460	1392

AGT CCT TCA GAA GAT AAT TTT ACT AAT GAT CTA AAT AAA GGA GAA GAA	1440
Ser Pro Ser Glu Asp Asn Phe Thr Asn Asp Leu Asn Lys Gly Glu Glu	
465 470 475 480	
ATT ACA TCT GAT ACT AAT ATA GAA GCA GCA GAA GAA AAT ATT AGT TTA	1488
Ile Thr Ser Asp Thr Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Leu	
485 490 495	
GAT TTA ATA CAA CAA TAT TAT TTA ACC TTT AAT TTT GAT AAT GAA CCT	1536
Asp Leu Ile Gln Gln Tyr Tyr Leu Thr Phe Asn Phe Asp Asn Glu Pro	
500 505 510	
GAA AAT ATT TCA ATA GAA AAT CTT TCA AGT GAC ATT ATA GGC CAA TTA	1584
Glu Asn Ile Ser Ile Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu	
515 520 525	
GAA CTT ATG CCT AAT ATA GAA AGA TTT CCT AAT GGA AAA AAG TAT GAG	1632
Glu Leu Met Pro Asn Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu	
530 535 540	
TTA GAT AAA TAT ACT ATG TTC CAT TAT CTT CGT GCT CAA GAA TTT GAA	1680
Leu Asp Lys Tyr Thr Met Phe His Tyr Leu Arg Ala Gln Glu Phe Glu	
545 550 555 560	
CAT GGT AAA TCT AGG ATT GCT TTA ACA AAT TCT GTT AAC GAA GCA TTA	1728
His Gly Lys Ser Arg Ile Ala Leu Thr Asn Ser Val Asn Glu Ala Leu	
565 570 575	
TTA AAT CCT AGT CGT GTT TAT ACA TTT TTT TCT TCA GAC TAT GTA AAG	1776
Leu Asn Pro Ser Arg Val Tyr Thr Phe Phe Ser Ser Asp Tyr Val Lys	
580 585 590	
AAA GTT AAT AAA GCT ACG GAG GCA GCT ATG TTT TTA GGC TGG GTA GAA	1824
Lys Val Asn Lys Ala Thr Glu Ala Ala Met Phe Leu Gly Trp Val Glu	
595 600 605	
CAA TTA GTA TAT GAT TTT ACC GAT GAA ACT AGC GAA GTA AGT ACT ACG	1872
Gln Leu Val Tyr Asp Phe Thr Asp Glu Thr Ser Glu Val Ser Thr Thr	
610 615 620	
GAT AAA ATT GCG GAT ATA ACT ATA ATT ATT CCA TAT ATA GGA CCT GCT	1920
Asp Lys Ile Ala Asp Ile Thr Ile Ile Ile Pro Tyr Ile Gly Pro Ala	
625 630 635 640	
TTA AAT ATA GGT AAT ATG TTA TAT AAA GAT GAT TTT GTA GGT GCT TTA	1968
Leu Asn Ile Gly Asn Met Leu Tyr Lys Asp Asp Phe Val Gly Ala Leu	
645 650 655	
ATA TTT TCA GGA GCT GTT ATT CTG TTA GAA TTT ATA CCA GAG ATT GCA	2016
Ile Phe Ser Gly Ala Val Ile Leu Leu Glu Phe Ile Pro Glu Ile Ala	
660 665 670	
ATA CCT GTA TTA GGT ACT TTT GCA CTT GTA TCA TAT ATT GCG AAT AAG	2064
Ile Pro Val Leu Gly Thr Phe Ala Leu Val Ser Tyr Ile Ala Asn Lys	
675 680 685	
GTT CTA ACC GTT CAA ACA ATA GAT AAT GCT TTA AGT AAA AGA AAT GAA	2112
Val Leu Thr Val Gln Thr Ile Asp Asn Ala Leu Ser Lys Arg Asn Glu	
690 695 700	

AAA TGG GAT GAG GTC TAT AAA TAT ATA GTA ACA AAT TGG TTA GCA AAG Lys Trp Asp Glu Val Tyr Lys Tyr Ile Val Thr Asn Trp Leu Ala Lys 705 710 715 720	2160
GTT AAT ACA CAG ATT GAT CTA ATA AGA AAA AAA ATG AAA GAA GCT TTA Val Asn Thr Gln Ile Asp Leu Ile Arg Lys Lys Met Lys Glu Ala Leu 725 730 735	2208
GAA AAT CAA GCA GAA GCA ACA AAG GCT ATA ATA AAC TAT CAG TAT AAT Glu Asn Gln Ala Glu Ala Thr Lys Ala Ile Ile Asn Tyr Gln Tyr Asn 740 745 750	2256
CAA TAT ACT GAG GAA GAG AAA AAT AAT ATT AAT TTT AAT ATT GAT GAT Gln Tyr Thr Glu Glu Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp 755 760 765	2304
TTA AGT TCG AAA CTT AAT GAG TCT ATA AAT AAA GCT ATG ATT AAT ATA Leu Ser Ser Lys Leu Asn Glu Ser Ile Asn Lys Ala Met Ile Asn Ile 770 775 780	2352
AAT AAA TTT TTG AAT CAA TGC TCT GTT TCA TAT TTA ATG AAT TCT ATG Asn Lys Phe Leu Asn Gln Cys Ser Val Ser Tyr Leu Met Asn Ser Met 785 790 795 800	2400
ATC CCT TAT GGT GTT AAA CGG TTA GAA GAT TTT GAT GCT AGT CTT AAA Ile Pro Tyr Gly Val Lys Arg Leu Glu Asp Phe Asp Ala Ser Leu Lys 805 810 815	2448
GAT GCA TTA TTA AAG TAT ATA TAT GAT AAT AGA GGA ACT TTA ATT GGT Asp Ala Leu Leu Lys Tyr Ile Tyr Asp Asn Arg Gly Thr Leu Ile Gly 820 825 830	2496
CAA GTA GAT AGA TTA AAA GAT AAA GTT AAT AAT ACA CTT AGT ACA GAT Gln Val Asp Arg Leu Lys Asp Lys Val Asn Asn Thr Leu Ser Thr Asp 835 840 845	2544
ATA CCT TTT CAG CTT TCC AAA TAC GTA GAT AAT CAA AGA TTA TTA TCT Ile Pro Phe Gln Leu Ser Lys Tyr Val Asp Asn Gln Arg Leu Leu Ser 850 855 860	2592
ACA TTT ACT GAA TAT ATT AAG AAT ATT ATT AAT ACT TCT ATA TTG AAT Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn 865 870 875 880	2640
TTA AGA TAT GAA AGT AAT CAT TTA ATA GAC TTA TCT AGG TAT GCA TCA Leu Arg Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser 885 890 895	2688
AAA ATA AAT ATT GGT AGT AAA GTA AAT TTT GAT CCA ATA GAT AAA AAT Lys Ile Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn 900 905 910	2736
CAA ATT CAA TTA TTT AAT TTA GAA AGT AGT AAA ATT GAG GTA ATT TTA Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu 915 920 925	2784
AAA AAT GCT ATT GTA TAT AAT AGT ATG TAT GAA AAT TTT AGT ACT AGC Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser 930 935 940	2832

TTT TGG ATA AGA ATT CCT AAG TAT TTT AAC AGT ATA AGT CTA AAT AAT Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn 945 950 955 960	2880
GAA TAT ACA ATA ATA AAT TGT ATG GAA AAT AAT TCA GGA TGG AAA GTA Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val 965 970 975	2928
TCA CTT AAT TAT GGT GAA ATA ATC TGG ACT TTA CAG GAT ACT CAG GAA Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu 980 985 990	2976
ATA AAA CAA AGA GTA GTT TTT AAA TAC AGT CAA ATG ATT AAT ATA TCA Ile Lys Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser 995 1000 1005	3024
GAT TAT ATA AAC AGA TGG ATT TTT GTA ACT ATC ACT AAT AAT AGA TTA Asp Tyr Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu 1010 1015 1020	3072
AAT AAC TCT AAA ATT TAT ATA AAT GGA AGA TTA ATA GAT CAA AAA CCA Asn Asn Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro 1025 1030 1035 1040	3120
ATT TCA AAT TTA GGT AAT ATT CAT GCT AGT AAT AAT ATA ATG TTT AAA Ile Ser Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys 1045 1050 1055	3168
TTA GAT GGT TGT AGA GAT ACA CAT AGA TAT ATT TGG ATA AAA TAT TTT Leu Asp Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe 1060 1065 1070	3216
AAT CTT TTT GAT AAG GAA TTA AAT GAA AAA GAA ATC AAA GAT TTA TAT Asn Leu Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr 1075 1080 1085	3264
GAT AAT CAA TCA AAT TCA GGT ATT TTA AAA GAC TTT TGG GGT GAT TAT Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr 1090 1095 1100	3312
TTA CAA TAT GAT AAA CCA TAC TAT ATG TTA AAT TTA TAT GAT CCA AAT Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn 1105 1110 1115 1120	3360
AAA TAT GTC GAT GTA AAT AAT GTA GGT ATT AGA GGT TAT ATG TAT CTT Lys Tyr Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu 1125 1130 1135	3408
AAA GGG CCT AGA GGT AGC GTA ATG ACT ACA AAC ATT TAT TTA AAT TCA Lys Gly Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser 1140 1145 1150	3456
AGT TTG TAT AGG GGG ACA AAA TTT ATT ATA AAA AAA TAT GCT TCT GGA Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly 1155 1160 1165	3504
AAT AAA GAT AAT ATT GTT AGA AAT AAT GAT CGT GTA TAT ATT AAT GTA Asn Lys Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val 1170 1175 1180	3552



GTA GTT AAA AAT AAA GAA TAT AGG TTA GCT ACT AAT GCA TCA CAG GCA Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala 1185 1190 1195 1200	3600
GGC GTA GAA AAA ATA CTA AGT GCA TTA GAA ATA CCT GAT GTA GGA AAT Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn 1205 1210 1215	3648
CTA AGT CAA GTA GTA GTA ATG AAG TCA AAA AAT GAT CAA GGA ATA ACA Leu Ser Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr 1220 1225 1230	3696
AAT AAA TGC AAA ATG AAT TTA CAA GAT AAT AAT GGG AAT GAT ATA GGC Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly 1235 1240 1245	3744
TTT ATA GGA TTT CAT CAG TTT AAT AAT ATA GCT AAA CTA GTA GCA AGT Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser 1250 1255 1260	3792
AAT TGG TAT AAT AGA CAA ATA GAA AGA TCT AGT AGG ACT TTG GGT TGC Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys 1265 1270 1275 1280	3840
TCA TGG GAA TTT ATT CCT GTA GAT GAT GGA TGG GGA GAA AGG CCA CTG Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu 1285 1290 1295	3888
TAA	3891

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1296 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Met Gln Phe Val Asn Lys Gln Phe Asn Tyr Lys Asp Pro Val Asn Gly 1 5 10 15
Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Val Gly Gln Met Gln Pro 20 25 30
Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val Ile Pro Glu Arg 35 40 45
Asp Thr Phe Thr Asn Pro Glu Glu Gly Asp Leu Asn Pro Pro Pro Glu 50 55 60
Ala Lys Gln Val Pro Val Ser Tyr Tyr Asp Ser Thr Tyr Leu Ser Thr 65 70 75 80
Asp Asn Glu Lys Asp Asn Tyr Leu Lys Gly Val Thr Lys Leu Phe Glu 85 90 95
Arg Ile Tyr Ser Thr Asp Leu Gly Arg Met Leu Leu Thr Ser Ile Val 100 105 110

Arg Gly Ile Pro Phe Trp Gly Gly Ser Thr Ile Asp Thr Glu Leu Lys  
 115 120 125  
 Val Ile Asp Thr Asn Cys Ile Asn Val Ile Gln Pro Asp Gly Ser Tyr  
 130 135 140  
 Arg Ser Glu Glu Leu Asn Leu Val Ile Ile Gly Pro Ser Ala Asp Ile  
 145 150 155 160  
 Ile Gln Phe Glu Cys Lys Ser Phe Gly His Glu Val Leu Asn Leu Thr  
 165 170 175  
 Arg Asn Gly Tyr Gly Ser Thr Gln Tyr Ile Arg Phe Ser Pro Asp Phe  
 180 185 190  
 Thr Phe Gly Phe Glu Glu Ser Leu Glu Val Asp Thr Asn Pro Leu Leu  
 195 200 205  
 Gly Ala Gly Lys Phe Ala Thr Asp Pro Ala Val Thr Leu Ala His Glu  
 210 215 220  
 Leu Ile His Ala Gly His Arg Leu Tyr Gly Ile Ala Ile Asn Pro Asn  
 225 230 235 240  
 Arg Val Phe Lys Val Asn Thr Asn Ala Tyr Tyr Glu Met Ser Gly Leu  
 245 250 255  
 Glu Val Ser Phe Glu Glu Leu Arg Thr Phe Gly Gly His Asp Ala Lys  
 260 265 270  
 Phe Ile Asp Ser Leu Gln Glu Asn Glu Phe Arg Leu Tyr Tyr Tyr Asn  
 275 280 285  
 Lys Phe Lys Asp Ile Ala Ser Thr Leu Asn Lys Ala Lys Ser Ile Val  
 290 295 300  
 Gly Thr Thr Ala Ser Leu Gln Tyr Met Lys Asn Val Phe Lys Glu Lys  
 305 310 315 320  
 Tyr Leu Leu Ser Glu Asp Thr Ser Gly Lys Phe Ser Val Asp Lys Leu  
 325 330 335  
 Lys Phe Asp Lys Leu Tyr Lys Met Leu Thr Glu Ile Tyr Thr Glu Asp  
 340 345 350  
 Asn Phe Val Lys Phe Phe Lys Val Leu Asn Arg Lys Thr Tyr Leu Asn  
 355 360 365  
 Phe Asp Lys Ala Val Phe Lys Ile Asn Ile Val Pro Lys Val Asn Tyr  
 370 375 380  
 Thr Ile Tyr Asp Gly Phe Asn Leu Arg Asn Thr Asn Leu Ala Ala Asn  
 385 390 395 400  
 Phe Asn Gly Gln Asn Thr Glu Ile Asn Asn Met Asn Phe Thr Lys Leu  
 405 410 415  
 Lys Asn Phe Thr Gly Leu Phe Glu Phe Tyr Lys Leu Leu Cys Val Arg  
 420 425 430  
 Gly Ile Ile Thr Ser Lys Thr Lys Ser Leu Asp Lys Gly Tyr Asn Lys  
 435 440 445

Ala Leu Asn Asp Leu Cys Ile Lys Val Asn Asn Trp Asp Leu Phe Phe  
 450 455 460  
 Ser Pro Ser Glu Asp Asn Phe Thr Asn Asp Leu Asn Lys Gly Glu Glu  
 465 470 475 480  
 Ile Thr Ser Asp Thr Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Leu  
 485 490 495  
 Asp Leu Ile Gln Gln Tyr Tyr Leu Thr Phe Asn Phe Asp Asn Glu Pro  
 500 505 510  
 Glu Asn Ile Ser Ile Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu  
 515 520 525  
 Glu Leu Met Pro Asn Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu  
 530 535 540  
 Leu Asp Lys Tyr Thr Met Phe His Tyr Leu Arg Ala Gln Glu Phe Glu  
 545 550 555 560  
 His Gly Lys Ser Arg Ile Ala Leu Thr Asn Ser Val Asn Glu Ala Leu  
 565 570 575  
 Leu Asn Pro Ser Arg Val Tyr Thr Phe Phe Ser Ser Asp Tyr Val Lys  
 580 585 590  
 Lys Val Asn Lys Ala Thr Glu Ala Ala Met Phe Leu Gly Trp Val Glu  
 595 600 605  
 Gln Leu Val Tyr Asp Phe Thr Asp Glu Thr Ser Glu Val Ser Thr Thr  
 610 615 620  
 Asp Lys Ile Ala Asp Ile Thr Ile Ile Ile Pro Tyr Ile Gly Pro Ala  
 625 630 635 640  
 Leu Asn Ile Gly Asn Met Leu Tyr Lys Asp Asp Phe Val Gly Ala Leu  
 645 650 655  
 Ile Phe Ser Gly Ala Val Ile Leu Leu Glu Phe Ile Pro Glu Ile Ala  
 660 665 670  
 Ile Pro Val Leu Gly Thr Phe Ala Leu Val Ser Tyr Ile Ala Asn Lys  
 675 680 685  
 Val Leu Thr Val Gln Thr Ile Asp Asn Ala Leu Ser Lys Arg Asn Glu  
 690 695 700  
 Lys Trp Asp Glu Val Tyr Lys Tyr Ile Val Thr Asn Trp Leu Ala Lys  
 705 710 715 720  
 Val Asn Thr Gln Ile Asp Leu Ile Arg Lys Lys Met Lys Glu Ala Leu  
 725 730 735  
 Glu Asn Gln Ala Glu Ala Thr Lys Ala Ile Ile Asn Tyr Gln Tyr Asn  
 740 745 750  
 Gln Tyr Thr Glu Glu Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp  
 755 760 765  
 Leu Ser Ser Lys Leu Asn Glu Ser Ile Asn Lys Ala Met Ile Asn Ile  
 770 775 780

Asn	Lys	Phe	Leu	Asn	Gln	Cys	Ser	Val	Ser	Tyr	Leu	Met	Asn	Ser	Met
785					790					795					800
Ile	Pro	Tyr	Gly	Val	Lys	Arg	Leu	Glu	Asp	Phe	Asp	Ala	Ser	Leu	Lys
				805					810					815	
Asp	Ala	Leu	Leu	Lys	Tyr	Ile	Tyr	Asp	Asn	Arg	Gly	Thr	Leu	Ile	Gly
				820				825					830		
Gln	Val	Asp	Arg	Leu	Lys	Asp	Lys	Val	Asn	Asn	Thr	Leu	Ser	Thr	Asp
		835					840					845			
Ile	Pro	Phe	Gln	Leu	Ser	Lys	Tyr	Val	Asp	Asn	Gln	Arg	Leu	Leu	Ser
	850					855					860				
Thr	Phe	Thr	Glu	Tyr	Ile	Lys	Asn	Ile	Ile	Asn	Thr	Ser	Ile	Leu	Asn
865					870					875					880
Leu	Arg	Tyr	Glu	Ser	Asn	His	Leu	Ile	Asp	Leu	Ser	Arg	Tyr	Ala	Ser
				885					890					895	
Lys	Ile	Asn	Ile	Gly	Ser	Lys	Val	Asn	Phe	Asp	Pro	Ile	Asp	Lys	Asn
			900					905					910		
Gln	Ile	Gln	Leu	Phe	Asn	Leu	Glu	Ser	Ser	Lys	Ile	Glu	Val	Ile	Leu
		915					920					925			
Lys	Asn	Ala	Ile	Val	Tyr	Asn	Ser	Met	Tyr	Glu	Asn	Phe	Ser	Thr	Ser
	930					935					940				
Phe	Trp	Ile	Arg	Ile	Pro	Lys	Tyr	Phe	Asn	Ser	Ile	Ser	Leu	Asn	Asn
945					950					955					960
Glu	Tyr	Thr	Ile	Ile	Asn	Cys	Met	Glu	Asn	Asn	Ser	Gly	Trp	Lys	Val
			965						970					975	
Ser	Leu	Asn	Tyr	Gly	Glu	Ile	Ile	Trp	Thr	Leu	Gln	Asp	Thr	Gln	Glu
			980					985					990		
Ile	Lys	Gln	Arg	Val	Val	Phe	Lys	Tyr	Ser	Gln	Met	Ile	Asn	Ile	Ser
		995					1000					1005			
Asp	Tyr	Ile	Asn	Arg	Trp	Ile	Phe	Val	Thr	Ile	Thr	Asn	Asn	Arg	Leu
	1010					1015					1020				
Asn	Asn	Ser	Lys	Ile	Tyr	Ile	Asn	Gly	Arg	Leu	Ile	Asp	Gln	Lys	Pro
1025					1030					1035					1040
Ile	Ser	Asn	Leu	Gly	Asn	Ile	His	Ala	Ser	Asn	Asn	Ile	Met	Phe	Lys
			1045					1050						1055	
Leu	Asp	Gly	Cys	Arg	Asp	Thr	His	Arg	Tyr	Ile	Trp	Ile	Lys	Tyr	Phe
		1060						1065					1070		
Asn	Leu	Phe	Asp	Lys	Glu	Leu	Asn	Glu	Lys	Glu	Ile	Lys	Asp	Leu	Tyr
		1075					1080					1085			
Asp	Asn	Gln	Ser	Asn	Ser	Gly	Ile	Leu	Lys	Asp	Phe	Trp	Gly	Asp	Tyr
	1090					1095					1100				
Leu	Gln	Tyr	Asp	Lys	Pro	Tyr	Tyr	Met	Leu	Asn	Leu	Tyr	Asp	Pro	Asn
1105					1110					1115					1120



(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1546 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACCATG GGCCATCATC	120
ATCATCATCA TCATCATCAT CACAGCAGCG GCCATATCGA AGGTCGTCAT ATGGCTAGCA	180
TGGCTAGATT ATTATCTACA TTTACTGAAT ATATTAAGAA TATTATTAAT ACTTCTATAT	240
TGAATTTAAG ATATGAAAGT AATCATTTAA TAGACTTATC TAGGTATGCA TCAAAAATAA	300
ATATTGGTAG TAAAGTAAAT TTTGATCCAA TAGATAAAAA TCAAATTCAA TTATTTAATT	360
TAGAAAGTAG TAAAATTGAG GTAATTTTAA AAAATGCTAT TGTATATAAT AGTATGTATG	420
AAAATTTTAG TACTAGCTTT TGGATAAGAA TTCCTAAGTA TTTTAACAGT ATAAGTCTAA	480
ATAATGAATA TACAATAATA AATTGTATGG AAAATAATTC AGGATGGAAA GTATCACTTA	540
ATTATGGTGA AATAATCTGG ACTTTACAGG AACTCAGGA AATAAAACAA AGAGTAGTTT	600
TTAAATACAG TCAAATGATT AATATATCAG ATTATATAAA CAGATGGATT TTTGTAACATA	660
TCACTAATAA TAGATTAAAT AACTCTAAAA TTTATATAAA TGGAAGATTA ATAGATCAAA	720
AACCAATTTT AAATTTAGGT AATATTCATG CTAGTAATAA TATAATGTTT AAATTAGATG	780
GTTGTAGAGA TACACATAGA TATATTTGGA TAAAATATTT TAATCTTTTT GATAAGGAAT	840
TAAATGAAAA AGAAATCAAA GATTTATATG ATAATCAATC AAATTCAGGT ATTTTAAAAG	900
ACTTTTGGGG TGATTATTTA CAATATGATA AACCATACTA TATGTTAAAT TTATATGATC	960
CAAATAAATA TGTCGATGTA AATAATGTAG GTATTAGAGG TTATATGTAT CTTAAAGGGC	1020
CTAGAGGTAG CGTAATGACT ACAAACATTT ATTTAAATTC AAGTTTGTAT AGGGGGACAA	1080
AATTTATTAT AAAAAAATAT GCTTCTGGAA ATAAAGATAA TATTGTTAGA AATAATGATC	1140
GTGTATATAT TAATGTAGTA GTTAAAAATA AAGAATATAG GTTAGCTACT AATGCATCAC	1200
AGGCAGGCGT AGAAAAAATA CTAAGTGCAT TAGAAATACC TGATGTAGGA AATCTAAGTC	1260
AAGTAGTAGT AATGAAGTCA AAAAATGATC AAGGAATAAC AAATAAATGC AAAATGAATT	1320
TACAAGATAA TAATGGGAAT GATATAGGCT TTATAGGATT TCATCAGTTT AATAATATAG	1380
CTAAACTAGT AGCAAGTAAT TGGTATAATA GACAAATAGA AAGATCTAGT AGGACTTTGG	1440

TTTGCTCATG GGAATTTATT CCTGTAGATG ATGGATGGGG AGAAAGGCCA CTGTAATTAA 1500

TCTCAAATA CATGAGTCTG TCAAGAAGCT TCGGCCGCA CTCGAG 1546

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 9 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Met His His His His His His Met Ala  
1 5

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TATGCATCAC CATCACCATC A 21

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CATGTGATGG TGATGGTGAT GCA 23

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1351 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "DNA"

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1335

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ATG CAT CAC CAT CAC CAT CAC ATG GCT CGT CTG CTG TCT ACC TTC ACT	48
Met His His His His His His Met Ala Arg Leu Leu Ser Thr Phe Thr	
1 5 10 15	
GAA TAC ATC AAG AAC ATC ATC AAT ACC TCC ATC CTG AAC CTG CGC TAC	96
Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg Tyr	
20 25 30	
GAA TCC AAT CAC CTG ATC GAC CTG TCT CGC TAC GCT TCC AAA ATC AAC	144
Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile Asn	
35 40 45	
ATC GGT TCT AAA GTT AAC TTC GAT CCG ATC GAC AAG AAT CAG ATC CAG	192
Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile Gln	
50 55 60	
CTG TTC AAT CTG GAA TCT TCC AAA ATC GAA GTT ATC CTG AAG AAT GCT	240
Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn Ala	
65 70 75 80	
ATC GTA TAC AAC TCT ATG TAC GAA AAC TTC TCC ACC TCC TTC TGG ATC	288
Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp Ile	
85 90 95	
CGT ATC CCG AAA TAC TTC AAC TCC ATC TCT CTG AAC AAT GAA TAC ACC	336
Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr Thr	
100 105 110	
ATC ATC AAC TGC ATG GAA AAC AAT TCT GGT TGG AAA GTA TCT CTG AAC	384
Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu Asn	
115 120 125	
TAC GGT GAA ATC ATC TGG ACT CTG CAG GAC ACT CAG GAA ATC AAA CAG	432
Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys Gln	
130 135 140	
CGT GTT GTA TTC AAA TAC TCT CAG ATG ATC AAC ATC TCT GAC TAC ATC	480
Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr Ile	
145 150 155 160	
AAT CGC TGG ATC TTC GTT ACC ATC ACC AAC AAT CGT CTG AAT AAC TCC	528
Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn Ser	
165 170 175	
AAA ATC TAC ATC AAC GGC CGT CTG ATC GAC CAG AAA CCG ATC TCC AAT	576
Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser Asn	
180 185 190	
CTG GGT AAC ATC CAC GCT TCT AAT AAC ATC ATG TTC AAA CTG GAC GGT	624
Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp Gly	
195 200 205	
TGT CGT GAC ACT CAC CGC TAC ATC TGG ATC AAA TAC TTC AAT CTG TTC	672
Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu Phe	
210 215 220	



GAC AAA GAA CTG AAC GAA AAA GAA ATC AAA GAC CTG TAC GAC AAC CAG Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn Gln 225 230 235 240	720
TCC AAT TCT GGT ATC CTG AAA GAC TTC TGG GGT GAC TAC CTG CAG TAC Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln Tyr 245 250 255	768
GAC AAA CCG TAC TAC ATG CTG AAT CTG TAC GAT CCG AAC AAA TAC GTT Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr Val 260 265 270	816
GAC GTC AAC AAT GTA GGT ATC CGC GGT TAC ATG TAC CTG AAA GGT CCG Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly Pro 275 280 285	864
CGT GGT TCT GTT ATG ACT ACC AAC ATC TAC CTG AAC TCT TCC CTG TAC Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu Tyr 290 295 300	912
CGT GGT ACC AAA TTC ATC ATC AAG AAA TAC GCG TCT GGT AAC AAG GAC Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys Asp 305 310 315 320	960
AAT ATC GTT CGC AAC AAT GAT CGT GTA TAC ATC AAT GTT GTA GTT AAG Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val Lys 325 330 335	1008
AAC AAA GAA TAC CGT CTG GCT ACC AAT GCT TCT CAG GCT GGT GTA GAA Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val Glu 340 345 350	1056
AAG ATC TTG TCT GCT CTG GAA ATC CCG GAC GTT GGT AAT CTG TCT CAG Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser Gln 355 360 365	1104
GTA GTT GTA ATG AAA TCC AAG AAC GAC CAG GGT ATC ACT AAC AAA TGC Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys Cys 370 375 380	1152
AAA ATG AAT CTG CAG GAC AAC AAT GGT AAC GAT ATC GGT TTC ATC GGT Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile Gly 385 390 395 400	1200
TTC CAC CAG TTC AAC AAT ATC GCT AAA CTG GTT GCT TCC AAC TGG TAC Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp Tyr 405 410 415	1248
AAT CGT CAG ATC GAA CGT TCC TCT CGC ACT CTG GGT TGC TCT TGG GAG Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp Glu 420 425 430	1296
TTC ATC CCG GTT GAT GAC GGT TGG GGT GAA CGT CCG CTG TAACCCGGGA Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu 435 440 445	1345
AAGCTT	1351

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 445 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Met His His His His His His Met Ala Arg Leu Leu Ser Thr Phe Thr  
1 5 10 15  
Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg Tyr  
20 25 30  
Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile Asn  
35 40 45  
Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile Gln  
50 55 60  
Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn Ala  
65 70 75 80  
Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp Ile  
85 90 95  
Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr Thr  
100 105 110  
Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu Asn  
115 120 125  
Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys Gln  
130 135 140  
Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr Ile  
145 150 155 160  
Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn Ser  
165 170 175  
Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser Asn  
180 185 190  
Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp Gly  
195 200 205  
Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu Phe  
210 215 220  
Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn Gln  
225 230 235 240  
Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln Tyr  
245 250 255  
Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr Val  
260 265 270

Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly Pro  
 275 280 285  
 Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu Tyr  
 290 295 300  
 Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys Asp  
 305 310 315 320  
 Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val Lys  
 325 330 335  
 Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val Glu  
 340 345 350  
 Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser Gln  
 355 360 365  
 Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys Cys  
 370 375 380  
 Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile Gly  
 385 390 395 400  
 Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp Tyr  
 405 410 415  
 Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp Glu  
 420 425 430  
 Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu  
 435 440 445

(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CGCATATGAA TATTCGTCCA TTGCATG

27

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GGAAGCTTGC AGGGCAATTA CATCATG

27

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3876 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..3873

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

ATG CCA GTT ACA ATA AAT AAT TTT AAT TAT AAT GAT CCT ATT GAT AAT	48
Met Pro Val Thr Ile Asn Asn Phe Asn Tyr Asn Asp Pro Ile Asp Asn	
1 5 10 15	
GAC AAT ATT ATT ATG ATG GAA CCT CCA TTT GCA AGG GGT ACG GGG AGA	96
Asp Asn Ile Ile Met Met Glu Pro Pro Phe Ala Arg Gly Thr Gly Arg	
20 25 30	
TAT TAT AAA GCT TTT AAA ATC ACA GAT CGT ATT TGG ATA ATA CCC GAA	144
Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Ile Pro Glu	
35 40 45	
AGA TAT ACT TTT GGA TAT AAA CCT GAG GAT TTT AAT AAA AGT TCC GGT	192
Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser Ser Gly	
50 55 60	
ATT TTT AAT AGA GAT GTT TGT GAA TAT TAT GAT CCA GAT TAC TTA AAT	240
Ile Phe Asn Arg Asp Val Cys Glu Tyr Tyr Asp Pro Asp Tyr Leu Asn	
65 70 75 80	
ACC AAT GAT AAA AAG AAT ATA TTT TTC CAA ACA TTG ATC AAG TTA TTT	288
Thr Asn Asp Lys Lys Asn Ile Phe Phe Gln Thr Leu Ile Lys Leu Phe	
85 90 95	
AAT AGA ATC AAA TCA AAA CCA TTG GGT GAA AAG TTA TTA GAG ATG ATT	336
Asn Arg Ile Lys Ser Lys Pro Leu Gly Glu Lys Leu Leu Glu Met Ile	
100 105 110	
ATA AAT GGT ATA CCT TAT CTT GGA GAT AGA CGT GTT CCA CTC GAA GAG	384
Ile Asn Gly Ile Pro Tyr Leu Gly Asp Arg Arg Val Pro Leu Glu Glu	
115 120 125	
TTT AAC ACA AAC ATT GCT AGT GTA ACT GTT AAT AAA TTA ATT AGT AAT	432
Phe Asn Thr Asn Ile Ala Ser Val Thr Val Asn Lys Leu Ile Ser Asn	
130 135 140	
CCA GGA GAA GTG GAG CGA AAA AAA GGT ATT TTC GCA AAT TTA ATA ATA	480
Pro Gly Glu Val Glu Arg Lys Lys Gly Ile Phe Ala Asn Leu Ile Ile	
145 150 155 160	
TTT GGA CCT GGG CCA GTT TTA AAT GAA AAT GAG ACT ATA GAT ATA GGT	528
Phe Gly Pro Gly Pro Val Leu Asn Glu Asn Glu Thr Ile Asp Ile Gly	
165 170 175	

ATA CAA AAT CAT TTT GCA TCA AGG GAA GGC TTT GGG GGT ATA ATG CAA Ile Gln Asn His Phe Ala Ser Arg Glu Gly Phe Gly Gly Ile Met Gln 180 185 190	576
ATG AAA TTT TGT CCA GAA TAT GTA AGC GTA TTT AAT AAT GTT CAA GAA Met Lys Phe Cys Pro Glu Tyr Val Ser Val Phe Asn Asn Val Gln Glu 195 200 205	624
AAC AAA GGC GCA AGT ATA TTT AAT AGA CGT GGA TAT TTT TCA GAT CCA Asn Lys Gly Ala Ser Ile Phe Asn Arg Arg Gly Tyr Phe Ser Asp Pro 210 215 220	672
GCC TTG ATA TTA ATG CAT GAA CTT ATA CAT GTT TTG CAT GGA TTA TAT Ala Leu Ile Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr 225 230 235 240	720
GGC ATT AAA GTA GAT GAT TTA CCA ATT GTA CCA AAT GAA AAA AAA TTT Gly Ile Lys Val Asp Asp Leu Pro Ile Val Pro Asn Glu Lys Lys Phe 245 250 255	768
TTT ATG CAA TCT ACA GAT ACT ATA CAG GCA GAA GAA CTA TAT ACA TTT Phe Met Gln Ser Thr Asp Thr Ile Gln Ala Glu Glu Leu Tyr Thr Phe 260 265 270	816
GGA GGA CAA GAT CCC AGC ATC ATA TCT CCT TCT ACA GAT AAA AGT ATC Gly Gly Gln Asp Pro Ser Ile Ile Ser Pro Ser Thr Asp Lys Ser Ile 275 280 285	864
TAT GAT AAA GTT TTG CAA AAT TTT AGG GGG ATA GTT GAT AGA CTT AAC Tyr Asp Lys Val Leu Gln Asn Phe Arg Gly Ile Val Asp Arg Leu Asn 290 295 300	912
AAG GTT TTA GTT TGC ATA TCA GAT CCT AAC ATT AAC ATT AAT ATA TAT Lys Val Leu Val Cys Ile Ser Asp Pro Asn Ile Asn Ile Asn Ile Tyr 305 310 315 320	960
AAA AAT AAA TTT AAA GAT AAA TAT AAA TTC GTT GAA GAT TCT GAA GGA Lys Asn Lys Phe Lys Asp Lys Tyr Lys Phe Val Glu Asp Ser Glu Gly 325 330 335	1008
AAA TAT AGT ATA GAT GTA GAA AGT TTC AAT AAA TTA TAT AAA AGC TTA Lys Tyr Ser Ile Asp Val Glu Ser Phe Asn Lys Leu Tyr Lys Ser Leu 340 345 350	1056
ATG TTA GGT TTT ACA GAA ATT AAT ATA GCA GAA AAT TAT AAA ATA AAA Met Leu Gly Phe Thr Glu Ile Asn Ile Ala Glu Asn Tyr Lys Ile Lys 355 360 365	1104
ACT AGA GCT TCT TAT TTT AGT GAT TCC TTA CCA CCA GTA AAA ATA AAA Thr Arg Ala Ser Tyr Phe Ser Asp Ser Leu Pro Pro Val Lys Ile Lys 370 375 380	1152
AAT TTA TTA GAT AAT GAA ATC TAT ACT ATA GAG GAA GGG TTT AAT ATA Asn Leu Leu Asp Asn Glu Ile Tyr Thr Ile Glu Glu Gly Phe Asn Ile 385 390 395 400	1200
TCT GAT AAA AAT ATG GGA AAA GAA TAT AGG GGT CAG AAT AAA GCT ATA Ser Asp Lys Asn Met Gly Lys Glu Tyr Arg Gly Gln Asn Lys Ala Ile 405 410 415	1248

AAT AAA CAA GCT TAT GAA GAA ATC AGC AAG GAG CAT TTG GCT GTA TAT	1296
Asn Lys Gln Ala Tyr Glu Glu Ile Ser Lys Glu His Leu Ala Val Tyr	
420 425 430	
AAG ATA CAA ATG TGT AAA AGT GTT AAA GTT CCA GGA ATA TGT ATT GAT	1344
Lys Ile Gln Met Cys Lys Ser Val Lys Val Pro Gly Ile Cys Ile Asp	
435 440 445	
GTC GAT AAT GAA AAT TTG TTC TTT ATA GCT GAT AAA AAT AGT TTT TCA	1392
Val Asp Asn Glu Asn Leu Phe Phe Ile Ala Asp Lys Asn Ser Phe Ser	
450 455 460	
GAT GAT TTA TCT AAA AAT GAA AGA GTA GAA TAT AAT ACA CAG AAT AAT	1440
Asp Asp Leu Ser Lys Asn Glu Arg Val Glu Tyr Asn Thr Gln Asn Asn	
465 470 475 480	
TAT ATA GGA AAT GAC TTT CCT ATA AAT GAA TTA ATT TTA GAT ACT GAT	1488
Tyr Ile Gly Asn Asp Phe Pro Ile Asn Glu Leu Ile Leu Asp Thr Asp	
485 490 495	
TTA ATA AGT AAA ATA GAA TTA CCA AGT GAA AAT ACA GAA TCA CTT ACT	1536
Leu Ile Ser Lys Ile Glu Leu Pro Ser Glu Asn Thr Glu Ser Leu Thr	
500 505 510	
GAT TTT AAT GTA GAT GTT CCA GTA TAT GAA AAA CAA CCC GCT ATA AAA	1584
Asp Phe Asn Val Asp Val Pro Val Tyr Glu Lys Gln Pro Ala Ile Lys	
515 520 525	
AAA GTT TTT ACA GAT GAA AAT ACC ATC TTT CAA TAT TTA TAC TCT CAG	1632
Lys Val Phe Thr Asp Glu Asn Thr Ile Phe Gln Tyr Leu Tyr Ser Gln	
530 535 540	
ACA TTT CCT CTA AAT ATA AGA GAT ATA AGT TTA ACA TCT TCA TTT GAT	1680
Thr Phe Pro Leu Asn Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp	
545 550 555 560	
GAT GCA TTA TTA GTT TCT AGC AAA GTT TAT TCA TTT TTT TCT ATG GAT	1728
Asp Ala Leu Leu Val Ser Ser Lys Val Tyr Ser Phe Phe Ser Met Asp	
565 570 575	
TAT ATT AAA ACT GCT AAT AAA GTA GTA GAA GCA GGA TTA TTT GCA GGT	1776
Tyr Ile Lys Thr Ala Asn Lys Val Val Glu Ala Gly Leu Phe Ala Gly	
580 585 590	
TGG GTG AAA CAG ATA GTA GAT GAT TTT GTA ATC GAA GCT AAT AAA AGC	1824
Trp Val Lys Gln Ile Val Asp Asp Phe Val Ile Glu Ala Asn Lys Ser	
595 600 605	
AGT ACT ATG GAT AAA ATT GCA GAT ATA TCT CTA ATT GTT CCT TAT ATA	1872
Ser Thr Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile	
610 615 620	
GGA TTA GCT TTA AAT GTA GGA GAT GAA ACA GCT AAA GGA AAT TTT GAA	1920
Gly Leu Ala Leu Asn Val Gly Asp Glu Thr Ala Lys Gly Asn Phe Glu	
625 630 635 640	
AGT GCT TTT GAG ATT GCA GGA TCC AGT ATT TTA CTA GAA TTT ATA CCA	1968
Ser Ala Phe Glu Ile Ala Gly Ser Ser Ile Leu Leu Glu Phe Ile Pro	
645 650 655	

GAA CTT TTA ATA CCT GTA GTT GGA GTC TTT TTA TTA GAA TCA TAT ATT Glu Leu Leu Ile Pro Val Val Gly Val Phe Leu Leu Glu Ser Tyr Ile 660 665 670	2016
GAC AAT AAA AAT AAA ATT ATT AAA ACA ATA GAT AAT GCT TTA ACT AAA Asp Asn Lys Asn Lys Ile Ile Lys Thr Ile Asp Asn Ala Leu Thr Lys 675 680 685	2064
AGA GTG GAA AAA TGG ATT GAT ATG TAC GGA TTA ATA GTA GCG CAA TGG Arg Val Glu Lys Trp Ile Asp Met Tyr Gly Leu Ile Val Ala Gln Trp 690 695 700	2112
CTC TCA ACA GTT AAT ACT CAA TTT TAT ACA ATA AAA GAG GGA ATG TAT Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile Lys Glu Gly Met Tyr 705 710 715 720	2160
AAG GCT TTA AAT TAT CAA GCA CAA GCA TTG GAA GAA ATA ATA AAA TAC Lys Ala Leu Asn Tyr Gln Ala Gln Ala Leu Glu Glu Ile Ile Lys Tyr 725 730 735	2208
AAA TAT AAT ATA TAT TCT GAA GAG GAA AAG TCA AAT ATT AAC ATC AAT Lys Tyr Asn Ile Tyr Ser Glu Glu Glu Lys Ser Asn Ile Asn Ile Asn 740 745 750	2256
TTT AAT GAT ATA AAT TCT AAA CTT AAT GAT GGT ATT AAC CAA GCT ATG Phe Asn Asp Ile Asn Ser Lys Leu Asn Asp Gly Ile Asn Gln Ala Met 755 760 765	2304
GAT AAT ATA AAT GAT TTT ATA AAT GAA TGT TCT GTA TCA TAT TTA ATG Asp Asn Ile Asn Asp Phe Ile Asn Glu Cys Ser Val Ser Tyr Leu Met 770 775 780	2352
AAA AAA ATG ATT CCA TTA GCT GTA AAA AAA TTA CTA GAC TTT GAT AAT Lys Lys Met Ile Pro Leu Ala Val Lys Lys Leu Leu Asp Phe Asp Asn 785 790 795 800	2400
ACT CTC AAA AAA AAT TTA TTA AAT TAT ATA GAT GAA AAT AAA TTA TAT Thr Leu Lys Lys Asn Leu Leu Asn Tyr Ile Asp Glu Asn Lys Leu Tyr 805 810 815	2448
TTA ATT GGA AGT GTA GAA GAT GAA AAA TCA AAA GTA GAT AAA TAC TTG Leu Ile Gly Ser Val Glu Asp Glu Lys Ser Lys Val Asp Lys Tyr Leu 820 825 830	2496
AAA ACC ATT ATA CCA TTT GAT CTT TCA ACG TAT TCT AAT ATT GAA ATA Lys Thr Ile Ile Pro Phe Asp Leu Ser Thr Tyr Ser Asn Ile Glu Ile 835 840 845	2544
CTA ATA AAA ATA TTT AAT AAA TAT AAT AGC GAA ATT TTA AAT AAT ATT Leu Ile Lys Ile Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile 850 855 860	2592
ATC TTA AAT TTA AGA TAT AGA GAT AAT AAT TTA ATA GAT TTA TCA GGA Ile Leu Asn Leu Arg Tyr Arg Asp Asn Asn Leu Ile Asp Leu Ser Gly 865 870 875 880	2640
TAT GGA GCA AAG GTA GAG GTA TAT GAT GGG GTC AAG CTT AAT GAT AAA Tyr Gly Ala Lys Val Glu Val Tyr Asp Gly Val Lys Leu Asn Asp Lys 885 890 895	2688

AAT CAA TTT AAA TTA ACT AGT TCA GCA GAT AGT AAG ATT AGA GTC ACT Asn Gln Phe Lys Leu Thr Ser Ser Ala Asp Ser Lys Ile Arg Val Thr 900 905 910	2736
CAA AAT CAG AAT ATT ATA TTT AAT AGT ATG TTC CTT GAT TTT AGC GTT Gln Asn Gln Asn Ile Ile Phe Asn Ser Met Phe Leu Asp Phe Ser Val 915 920 925	2784
AGC TTT TGG ATA AGG ATA CCT AAA TAT AGG AAT GAT GAT ATA CAA AAT Ser Phe Trp Ile Arg Ile Pro Lys Tyr Arg Asn Asp Asp Ile Gln Asn 930 935 940	2832
TAT ATT CAT AAT GAA TAT ACG ATA ATT AAT TGT ATG AAA AAT AAT TCA Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser 945 950 955 960	2880
GGC TGG AAA ATA TCT ATT AGG GGT AAT AGG ATA ATA TGG ACC TTA ATT Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile 965 970 975	2928
GAT ATA AAT GGA AAA ACC AAA TCA GTA TTT TTT GAA TAT AAC ATA AGA Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg 980 985 990	2976
GAA GAT ATA TCA GAG TAT ATA AAT AGA TGG TTT TTT GTA ACT ATT ACT Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr 995 1000 1005	3024
AAT AAT TTG GAT AAT GCT AAA ATT TAT ATT AAT GGC ACG TTA GAA TCA Asn Asn Leu Asp Asn Ala Lys Ile Tyr Ile Asn Gly Thr Leu Glu Ser 1010 1015 1020	3072
AAT ATG GAT ATT AAA GAT ATA GGA GAA GTT ATT GTT AAT GGT GAA ATA Asn Met Asp Ile Lys Asp Ile Gly Glu Val Ile Val Asn Gly Glu Ile 1025 1030 1035 1040	3120
ACA TTT AAA TTA GAT GGT GAT GTA GAT AGA ACA CAA TTT ATT TGG ATG Thr Phe Lys Leu Asp Gly Asp Val Asp Arg Thr Gln Phe Ile Trp Met 1045 1050 1055	3168
AAA TAT TTT AGT ATT TTT AAT ACG CAA TTA AAT CAA TCA AAT ATT AAA Lys Tyr Phe Ser Ile Phe Asn Thr Gln Leu Asn Gln Ser Asn Ile Lys 1060 1065 1070	3216
GAG ATA TAT AAA ATT CAA TCA TAT AGC GAA TAC TTA AAA GAT TTT TGG Glu Ile Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp 1075 1080 1085	3264
GGA AAT CCT TTA ATG TAT AAT AAA GAA TAT TAT ATG TTT AAT GCG GGG Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly 1090 1095 1100	3312
AAT AAA AAT TCA TAT ATT AAA CTA GTG AAA GAT TCA TCT GTA GGT GAA Asn Lys Asn Ser Tyr Ile Lys Leu Val Lys Asp Ser Ser Val Gly Glu 1105 1110 1115 1120	3360
ATA TTA ATA CGT AGC AAA TAT AAT CAG AAT TCC AAT TAT ATA AAT TAT Ile Leu Ile Arg Ser Lys Tyr Asn Gln Asn Ser Asn Tyr Ile Asn Tyr 1125 1130 1135	3408



AGA AAT TTA TAT ATT GGA GAA AAA TTT ATT ATA AGA AGA GAG TCA AAT Arg Asn Leu Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Glu Ser Asn 1140 1145 1150	3456
TCT CAA TCT ATA AAT GAT GAT ATA GTT AGA AAA GAA GAT TAT ATA CAT Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile His 1155 1160 1165	3504
CTA GAT TTG GTA CTT CAC CAT GAA GAG TGG AGA GTA TAT GCC TAT AAA Leu Asp Leu Val Leu His His Glu Glu Trp Arg Val Tyr Ala Tyr Lys 1170 1175 1180	3552
TAT TTT AAG GAA CAG GAA GAA AAA TTG TTT TTA TCT ATT ATA AGT GAT Tyr Phe Lys Glu Gln Glu Glu Lys Leu Phe Leu Ser Ile Ile Ser Asp 1185 1190 1195 1200	3600
TCT AAT GAA TTT TAT AAG ACT ATA GAA ATA AAA GAA TAT GAT GAA CAG Ser Asn Glu Phe Tyr Lys Thr Ile Glu Ile Lys Glu Tyr Asp Glu Gln 1205 1210 1215	3648
CCA TCA TAT AGT TGT CAG TTG CTT TTT AAA AAA GAT GAA GAA AGT ACT Pro Ser Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr 1220 1225 1230	3696
GAT GAT ATA GGA TTG ATT GGT ATT CAT CGT TTC TAC GAA TCT GGA GTT Asp Asp Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Val 1235 1240 1245	3744
TTA CGT AAA AAG TAT AAA GAT TAT TTT TGT ATA AGT AAA TGG TAC TTA Leu Arg Lys Lys Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu 1250 1255 1260	3792
AAA GAG GTA AAA AGG AAA CCA TAT AAG TCA AAT TTG GGA TGT AAT TGG Lys Glu Val Lys Arg Lys Pro Tyr Lys Ser Asn Leu Gly Cys Asn Trp 1265 1270 1275 1280	3840
CAG TTT ATT CCT AAA GAT GAA GGG TGG ACT GAA TAA Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu 1285 1290	3876

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1291 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Met Pro Val Thr Ile Asn Asn Phe Asn Tyr Asn Asp Pro Ile Asp Asn 1 5 10 15
Asp Asn Ile Ile Met Met Glu Pro Pro Phe Ala Arg Gly Thr Gly Arg 20 25 30
Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Ile Pro Glu 35 40 45
Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser Ser Gly 50 55 60

Ile	Phe	Asn	Arg	Asp	Val	Cys	Glu	Tyr	Tyr	Asp	Pro	Asp	Tyr	Leu	Asn	65	70	75	80
Thr	Asn	Asp	Lys	Lys	Asn	Ile	Phe	Phe	Gln	Thr	Leu	Ile	Lys	Leu	Phe	85	90	95	
Asn	Arg	Ile	Lys	Ser	Lys	Pro	Leu	Gly	Glu	Lys	Leu	Leu	Glu	Met	Ile	100	105	110	
Ile	Asn	Gly	Ile	Pro	Tyr	Leu	Gly	Asp	Arg	Arg	Val	Pro	Leu	Glu	Glu	115	120	125	
Phe	Asn	Thr	Asn	Ile	Ala	Ser	Val	Thr	Val	Asn	Lys	Leu	Ile	Ser	Asn	130	135	140	
Pro	Gly	Glu	Val	Glu	Arg	Lys	Lys	Gly	Ile	Phe	Ala	Asn	Leu	Ile	Ile	145	150	155	160
Phe	Gly	Pro	Gly	Pro	Val	Leu	Asn	Glu	Asn	Glu	Thr	Ile	Asp	Ile	Gly	165	170	175	
Ile	Gln	Asn	His	Phe	Ala	Ser	Arg	Glu	Gly	Phe	Gly	Gly	Ile	Met	Gln	180	185	190	
Met	Lys	Phe	Cys	Pro	Glu	Tyr	Val	Ser	Val	Phe	Asn	Asn	Val	Gln	Glu	195	200	205	
Asn	Lys	Gly	Ala	Ser	Ile	Phe	Asn	Arg	Arg	Gly	Tyr	Phe	Ser	Asp	Pro	210	215	220	
Ala	Leu	Ile	Leu	Met	His	Glu	Leu	Ile	His	Val	Leu	His	Gly	Leu	Tyr	225	230	235	240
Gly	Ile	Lys	Val	Asp	Asp	Leu	Pro	Ile	Val	Pro	Asn	Glu	Lys	Lys	Phe	245	250	255	
Phe	Met	Gln	Ser	Thr	Asp	Thr	Ile	Gln	Ala	Glu	Glu	Leu	Tyr	Thr	Phe	260	265	270	
Gly	Gly	Gln	Asp	Pro	Ser	Ile	Ile	Ser	Pro	Ser	Thr	Asp	Lys	Ser	Ile	275	280	285	
Tyr	Asp	Lys	Val	Leu	Gln	Asn	Phe	Arg	Gly	Ile	Val	Asp	Arg	Leu	Asn	290	295	300	
Lys	Val	Leu	Val	Cys	Ile	Ser	Asp	Pro	Asn	Ile	Asn	Ile	Asn	Ile	Tyr	305	310	315	320
Lys	Asn	Lys	Phe	Lys	Asp	Lys	Tyr	Lys	Phe	Val	Glu	Asp	Ser	Glu	Gly	325	330	335	
Lys	Tyr	Ser	Ile	Asp	Val	Glu	Ser	Phe	Asn	Lys	Leu	Tyr	Lys	Ser	Leu	340	345	350	
Met	Leu	Gly	Phe	Thr	Glu	Ile	Asn	Ile	Ala	Glu	Asn	Tyr	Lys	Ile	Lys	355	360	365	
Thr	Arg	Ala	Ser	Tyr	Phe	Ser	Asp	Ser	Leu	Pro	Pro	Val	Lys	Ile	Lys	370	375	380	
Asn	Leu	Leu	Asp	Asn	Glu	Ile	Tyr	Thr	Ile	Glu	Glu	Gly	Phe	Asn	Ile	385	390	395	400

Ser Asp Lys Asn Met Gly Lys Glu Tyr Arg Gly Gln Asn Lys Ala Ile  
 405 410 415  
 Asn Lys Gln Ala Tyr Glu Glu Ile Ser Lys Glu His Leu Ala Val Tyr  
 420 425 430  
 Lys Ile Gln Met Cys Lys Ser Val Lys Val Pro Gly Ile Cys Ile Asp  
 435 440 445  
 Val Asp Asn Glu Asn Leu Phe Phe Ile Ala Asp Lys Asn Ser Phe Ser  
 450 455 460  
 Asp Asp Leu Ser Lys Asn Glu Arg Val Glu Tyr Asn Thr Gln Asn Asn  
 465 470 475 480  
 Tyr Ile Gly Asn Asp Phe Pro Ile Asn Glu Leu Ile Leu Asp Thr Asp  
 485 490 495  
 Leu Ile Ser Lys Ile Glu Leu Pro Ser Glu Asn Thr Glu Ser Leu Thr  
 500 505 510  
 Asp Phe Asn Val Asp Val Pro Val Tyr Glu Lys Gln Pro Ala Ile Lys  
 515 520 525  
 Lys Val Phe Thr Asp Glu Asn Thr Ile Phe Gln Tyr Leu Tyr Ser Gln  
 530 535 540  
 Thr Phe Pro Leu Asn Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp  
 545 550 555 560  
 Asp Ala Leu Leu Val Ser Ser Lys Val Tyr Ser Phe Phe Ser Met Asp  
 565 570 575  
 Tyr Ile Lys Thr Ala Asn Lys Val Val Glu Ala Gly Leu Phe Ala Gly  
 580 585 590  
 Trp Val Lys Gln Ile Val Asp Asp Phe Val Ile Glu Ala Asn Lys Ser  
 595 600 605  
 Ser Thr Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile  
 610 615 620  
 Gly Leu Ala Leu Asn Val Gly Asp Glu Thr Ala Lys Gly Asn Phe Glu  
 625 630 635 640  
 Ser Ala Phe Glu Ile Ala Gly Ser Ser Ile Leu Leu Glu Phe Ile Pro  
 645 650 655  
 Glu Leu Leu Ile Pro Val Val Gly Val Phe Leu Leu Glu Ser Tyr Ile  
 660 665 670  
 Asp Asn Lys Asn Lys Ile Ile Lys Thr Ile Asp Asn Ala Leu Thr Lys  
 675 680 685  
 Arg Val Glu Lys Trp Ile Asp Met Tyr Gly Leu Ile Val Ala Gln Trp  
 690 695 700  
 Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile Lys Glu Gly Met Tyr  
 705 710 715 720  
 Lys Ala Leu Asn Tyr Gln Ala Gln Ala Leu Glu Glu Ile Ile Lys Tyr  
 725 730 735

Lys Tyr Asn Ile Tyr Ser Glu Glu Glu Lys Ser Asn Ile Asn Ile Asn  
 740 745 750  
 Phe Asn Asp Ile Asn Ser Lys Leu Asn Asp Gly Ile Asn Gln Ala Met  
 755 760 765  
 Asp Asn Ile Asn Asp Phe Ile Asn Glu Cys Ser Val Ser Tyr Leu Met  
 770 775 780  
 Lys Lys Met Ile Pro Leu Ala Val Lys Lys Leu Leu Asp Phe Asp Asn  
 785 790 795 800  
 Thr Leu Lys Lys Asn Leu Leu Asn Tyr Ile Asp Glu Asn Lys Leu Tyr  
 805 810 815  
 Leu Ile Gly Ser Val Glu Asp Glu Lys Ser Lys Val Asp Lys Tyr Leu  
 820 825 830  
 Lys Thr Ile Ile Pro Phe Asp Leu Ser Thr Tyr Ser Asn Ile Glu Ile  
 835 840 845  
 Leu Ile Lys Ile Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile  
 850 855 860  
 Ile Leu Asn Leu Arg Tyr Arg Asp Asn Asn Leu Ile Asp Leu Ser Gly  
 865 870 875 880  
 Tyr Gly Ala Lys Val Glu Val Tyr Asp Gly Val Lys Leu Asn Asp Lys  
 885 890 895  
 Asn Gln Phe Lys Leu Thr Ser Ser Ala Asp Ser Lys Ile Arg Val Thr  
 900 905 910  
 Gln Asn Gln Asn Ile Ile Phe Asn Ser Met Phe Leu Asp Phe Ser Val  
 915 920 925  
 Ser Phe Trp Ile Arg Ile Pro Lys Tyr Arg Asn Asp Asp Ile Gln Asn  
 930 935 940  
 Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser  
 945 950 955 960  
 Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile  
 965 970 975  
 Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg  
 980 985 990  
 Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr  
 995 1000 1005  
 Asn Asn Leu Asp Asn Ala Lys Ile Tyr Ile Asn Gly Thr Leu Glu Ser  
 1010 1015 1020  
 Asn Met Asp Ile Lys Asp Ile Gly Glu Val Ile Val Asn Gly Glu Ile  
 1025 1030 1035 1040  
 Thr Phe Lys Leu Asp Gly Asp Val Asp Arg Thr Gln Phe Ile Trp Met  
 1045 1050 1055  
 Lys Tyr Phe Ser Ile Phe Asn Thr Gln Leu Asn Gln Ser Asn Ile Lys  
 1060 1065 1070

Glu Ile Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp  
 1075 1080 1085  
 Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly  
 1090 1095 1100  
 Asn Lys Asn Ser Tyr Ile Lys Leu Val Lys Asp Ser Ser Val Gly Glu  
 1105 1110 1115 1120  
 Ile Leu Ile Arg Ser Lys Tyr Asn Gln Asn Ser Asn Tyr Ile Asn Tyr  
 1125 1130 1135  
 Arg Asn Leu Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Glu Ser Asn  
 1140 1145 1150  
 Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile His  
 1155 1160 1165  
 Leu Asp Leu Val Leu His His Glu Glu Trp Arg Val Tyr Ala Tyr Lys  
 1170 1175 1180  
 Tyr Phe Lys Glu Gln Glu Glu Lys Leu Phe Leu Ser Ile Ile Ser Asp  
 1185 1190 1195 1200  
 Ser Asn Glu Phe Tyr Lys Thr Ile Glu Ile Lys Glu Tyr Asp Glu Gln  
 1205 1210 1215  
 Pro Ser Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr  
 1220 1225 1230  
 Asp Asp Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Val  
 1235 1240 1245  
 Leu Arg Lys Lys Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu  
 1250 1255 1260  
 Lys Glu Val Lys Arg Lys Pro Tyr Lys Ser Asn Leu Gly Cys Asn Trp  
 1265 1270 1275 1280  
 Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu  
 1285 1290

(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 3876 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
- (A) NAME/KEY: CDS
  - (B) LOCATION: 1..3873

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

ATG CCA GTT ACA ATA AAT AAT TTT AAT TAT AAT GAT CCT ATT GAT AAT  
 Met Pro Val Thr Ile Asn Asn Phe Asn Tyr Asn Asp Pro Ile Asp Asn  
 1 5 10 15

48

AAT AAT ATT ATT ATG ATG GAG CCT CCA TTT GCG AGA GGT ACG GGG AGA Asn Asn Ile Ile Met Met Glu Pro Pro Phe Ala Arg Gly Thr Gly Arg 20 25 30	96
TAT TAT AAA GCT TTT AAA ATC ACA GAT CGT ATT TGG ATA ATA CCG GAA Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Ile Pro Glu 35 40 45	144
AGA TAT ACT TTT GGA TAT AAA CCT GAG GAT TTT AAT AAA AGT TCC GGT Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser Ser Gly 50 55 60	192
ATT TTT AAT AGA GAT GTT TGT GAA TAT TAT GAT CCA GAT TAC TTA AAT Ile Phe Asn Arg Asp Val Cys Glu Tyr Tyr Asp Pro Asp Tyr Leu Asn 65 70 75 80	240
ACT AAT GAT AAA AAG AAT ATA TTT TTA CAA ACA ATG ATC AAG TTA TTT Thr Asn Asp Lys Lys Asn Ile Phe Leu Gln Thr Met Ile Lys Leu Phe 85 90 95	288
AAT AGA ATC AAA TCA AAA CCA TTG GGT GAA AAG TTA TTA GAG ATG ATT Asn Arg Ile Lys Ser Lys Pro Leu Gly Glu Lys Leu Leu Glu Met Ile 100 105 110	336
ATA AAT GGT ATA CCT TAT CTT GGA GAT AGA CGT GTT CCA CTC GAA GAG Ile Asn Gly Ile Pro Tyr Leu Gly Asp Arg Arg Val Pro Leu Glu Glu 115 120 125	384
TTT AAC ACA AAC ATT GCT AGT GTA ACT GTT AAT AAA TTA ATC AGT AAT Phe Asn Thr Asn Ile Ala Ser Val Thr Val Asn Lys Leu Ile Ser Asn 130 135 140	432
CCA GGA GAA GTG GAG CGA AAA AAA GGT ATT TTC GCA AAT TTA ATA ATA Pro Gly Glu Val Glu Arg Lys Lys Gly Ile Phe Ala Asn Leu Ile Ile 145 150 155 160	480
TTT GGA CCT GGG CCA GTT TTA AAT GAA AAT GAG ACT ATA GAT ATA GGT Phe Gly Pro Gly Pro Val Leu Asn Glu Asn Glu Thr Ile Asp Ile Gly 165 170 175	528
ATA CAA AAT CAT TTT GCA TCA AGG GAA GGC TTC GGG GGT ATA ATG CAA Ile Gln Asn His Phe Ala Ser Arg Glu Gly Phe Gly Gly Ile Met Gln 180 185 190	576
ATG AAG TTT TGC CCA GAA TAT GTA AGC GTA TTT AAT AAT GTT CAA GAA Met Lys Phe Cys Pro Glu Tyr Val Ser Val Phe Asn Asn Val Gln Glu 195 200 205	624
AAC AAA GGC GCA AGT ATA TTT AAT AGA CGT GGA TAT TTT TCA GAT CCA Asn Lys Gly Ala Ser Ile Phe Asn Arg Arg Gly Tyr Phe Ser Asp Pro 210 215 220	672
GCC TTG ATA TTA ATG CAT GAA CTT ATA CAT GTT TTA CAT GGA TTA TAT Ala Leu Ile Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr 225 230 235 240	720
GGC ATT AAA GTA GAT GAT TTA CCA ATT GTA CCA AAT GAA AAA AAA TTT Gly Ile Lys Val Asp Asp Leu Pro Ile Val Pro Asn Glu Lys Lys Phe 245 250 255	768

TTT ATG CAA TCT ACA GAT GCT ATA CAG GCA GAA GAA CTA TAT ACA TTT	816
Phe Met Gln Ser Thr Asp Ala Ile Gln Ala Glu Glu Leu Tyr Thr Phe	
260 265 270	
GGA GGA CAA GAT CCC AGC ATC ATA ACT CCT TCT ACG GAT AAA AGT ATC	864
Gly Gly Gln Asp Pro Ser Ile Ile Thr Pro Ser Thr Asp Lys Ser Ile	
275 280 285	
TAT GAT AAA GTT TTG CAA AAT TTT AGA GGG ATA GTT GAT AGA CTT AAC	912
Tyr Asp Lys Val Leu Gln Asn Phe Arg Gly Ile Val Asp Arg Leu Asn	
290 295 300	
AAG GTT TTA GTT TGC ATA TCA GAT CCT AAC ATT AAT ATT AAT ATA TAT	960
Lys Val Leu Val Cys Ile Ser Asp Pro Asn Ile Asn Ile Asn Ile Tyr	
305 310 315 320	
AAA AAT AAA TTT AAA GAT AAA TAT AAA TTC GTT GAA GAT TCT GAG GGA	1008
Lys Asn Lys Phe Lys Asp Lys Tyr Lys Phe Val Glu Asp Ser Glu Gly	
325 330 335	
AAA TAT AGT ATA GAT GTA GAA AGT TTT GAT AAA TTA TAT AAA AGC TTA	1056
Lys Tyr Ser Ile Asp Val Glu Ser Phe Asp Lys Leu Tyr Lys Ser Leu	
340 345 350	
ATG TTT GGT TTT ACA GAA ACT AAT ATA GCA GAA AAT TAT AAA ATA AAA	1104
Met Phe Gly Phe Thr Glu Thr Asn Ile Ala Glu Asn Tyr Lys Ile Lys	
355 360 365	
ACT AGA GCT TCT TAT TTT AGT GAT TCC TTA CCA CCA GTA AAA ATA AAA	1152
Thr Arg Ala Ser Tyr Phe Ser Asp Ser Leu Pro Pro Val Lys Ile Lys	
370 375 380	
AAT TTA TTA GAT AAT GAA ATC TAT ACT ATA GAG GAA GGG TTT AAT ATA	1200
Asn Leu Leu Asp Asn Glu Ile Tyr Thr Ile Glu Glu Gly Phe Asn Ile	
385 390 395 400	
TCT GAT AAA GAT ATG GAA AAA GAA TAT AGA GGT CAG AAT AAA GCT ATA	1248
Ser Asp Lys Asp Met Glu Lys Glu Tyr Arg Gly Gln Asn Lys Ala Ile	
405 410 415	
AAT AAA CAA GCT TAT GAA GAA ATT AGC AAG GAG CAT TTG GCT GTA TAT	1296
Asn Lys Gln Ala Tyr Glu Glu Ile Ser Lys Glu His Leu Ala Val Tyr	
420 425 430	
AAG ATA CAA ATG TGT AAA AGT GTT AAA GCT CCA GGA ATA TGT ATT GAT	1344
Lys Ile Gln Met Cys Lys Ser Val Lys Ala Pro Gly Ile Cys Ile Asp	
435 440 445	
GTT GAT AAT GAA GAT TTG TTC TTT ATA GCT GAT AAA AAT AGT TTT TCA	1392
Val Asp Asn Glu Asp Leu Phe Phe Ile Ala Asp Lys Asn Ser Phe Ser	
450 455 460	
GAT GAT TTA TCT AAA AAC GAA AGA ATA GAA TAT AAT ACA CAG AGT AAT	1440
Asp Asp Leu Ser Lys Asn Glu Arg Ile Glu Tyr Asn Thr Gln Ser Asn	
465 470 475 480	
TAT ATA GAA AAT GAC TTC CCT ATA AAT GAA TTA ATT TTA GAT ACT GAT	1488
Tyr Ile Glu Asn Asp Phe Pro Ile Asn Glu Leu Ile Leu Asp Thr Asp	
485 490 495	

TTA ATA AGT AAA ATA GAA TTA CCA AGT GAA AAT ACA GAA TCA CTT ACT Leu Ile Ser Lys Ile Glu Leu Pro Ser Glu Asn Thr Glu Ser Leu Thr 500 505 510	1536
GAT TTT AAT GTA GAT GTT CCA GTA TAT GAA AAA CAA CCC GCT ATA AAA Asp Phe Asn Val Asp Val Pro Val Tyr Glu Lys Gln Pro Ala Ile Lys 515 520 525	1584
AAA ATT TTT ACA GAT GAA AAT ACC ATC TTT CAA TAT TTA TAC TCT CAG Lys Ile Phe Thr Asp Glu Asn Thr Ile Phe Gln Tyr Leu Tyr Ser Gln 530 535 540	1632
ACA TTT CTC TTA GAT ATA AGA GAT ATA AGT TTA ACA TCT TCA TTT GAT Thr Phe Leu Leu Asp Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp 545 550 555 560	1680
GAT GCA TTA TTA TTT TCT AAC AAA GTT TAT TCA TTT TTT TCT ATG GAT Asp Ala Leu Leu Phe Ser Asn Lys Val Tyr Ser Phe Phe Ser Met Asp 565 570 575	1728
TAT ATT AAA ACT GCT AAT AAA GTG GTA GAA GCA GGA TTA TTT GCA GGT Tyr Ile Lys Thr Ala Asn Lys Val Val Glu Ala Gly Leu Phe Ala Gly 580 585 590	1776
TGG GTG AAA CAG ATA GTA AAT GAT TTT GTA ATC GAA GCT AAT AAA AGC Trp Val Lys Gln Ile Val Asn Asp Phe Val Ile Glu Ala Asn Lys Ser 595 600 605	1824
AAT ACT ATG GAT AAA ATT GCA GAT ATA TCT CTA ATT GTT CCT TAT ATA Asn Thr Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile 610 615 620	1872
GGA TTA GCT TTA AAT GTA GGA AAT GAA ACA GCT AAA GGA AAT TTT GAA Gly Leu Ala Leu Asn Val Gly Asn Glu Thr Ala Lys Gly Asn Phe Glu 625 630 635 640	1920
AAT GCT TTT GAG ATT GCA GGA GCC AGT ATT CTA CTA GAA TTT ATA CCA Asn Ala Phe Glu Ile Ala Gly Ala Ser Ile Leu Leu Glu Phe Ile Pro 645 650 655	1968
GAA CTT TTA ATA CCT GTA GTT GGA GCC TTT TTA TTA GAA TCA TAT ATT Glu Leu Leu Ile Pro Val Val Gly Ala Phe Leu Leu Glu Ser Tyr Ile 660 665 670	2016
GAC AAT AAA AAT AAA ATT ATT AAA ACA ATA GAT AAT GCT TTA ACT AAA Asp Asn Lys Asn Lys Ile Ile Lys Thr Ile Asp Asn Ala Leu Thr Lys 675 680 685	2064
AGA AAT GAA AAA TGG AGT GAT ATG TAC GGA TTA ATA GTA GCG CAA TGG Arg Asn Glu Lys Trp Ser Asp Met Tyr Gly Leu Ile Val Ala Gln Trp 690 695 700	2112
CTC TCA ACA GTT AAT ACT CAA TTT TAT ACA ATA AAA GAG GGA ATG TAT Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile Lys Glu Gly Met Tyr 705 710 715 720	2160
AAG GCT TTA AAT TAT CAA GCA CAA GCA TTG GAA GAA ATA ATA AAA TAC Lys Ala Leu Asn Tyr Gln Ala Gln Ala Leu Glu Glu Ile Ile Lys Tyr 725 730 735	2208



AGA TAT AAT ATA TAT TCT GAA AAA GAA AAG TCA AAT ATT AAC ATC GAT Arg Tyr Asn Ile Tyr Ser Glu Lys Glu Lys Ser Asn Ile Asn Ile Asp 740 745 750	2256
TTT AAT GAT ATA AAT TCT AAA CTT AAT GAG GGT ATT AAC CAA GCT ATA Phe Asn Asp Ile Asn Ser Lys Leu Asn Glu Gly Ile Asn Gln Ala Ile 755 760 765	2304
GAT AAT ATA AAT AAT TTT ATA AAT GGA TGT TCT GTA TCA TAT TTA ATG Asp Asn Ile Asn Asn Phe Ile Asn Gly Cys Ser Val Ser Tyr Leu Met 770 775 780	2352
AAA AAA ATG ATT CCA TTA GCT GTA GAA AAA TTA CTA GAC TTT GAT AAT Lys Lys Met Ile Pro Leu Ala Val Glu Lys Leu Leu Asp Phe Asp Asn 785 790 795 800	2400
ACT CTC AAA AAA AAT TTG TTA AAT TAT ATA GAT GAA AAT AAA TTA TAT Thr Leu Lys Lys Asn Leu Leu Asn Tyr Ile Asp Glu Asn Lys Leu Tyr 805 810 815	2448
TTG ATT GGA AGT GCA GAA TAT GAA AAA TCA AAA GTA AAT AAA TAC TTG Leu Ile Gly Ser Ala Glu Tyr Glu Lys Ser Lys Val Asn Lys Tyr Leu 820 825 830	2496
AAA ACC ATT ATG CCG TTT GAT CTT TCA ATA TAT ACC AAT GAT ACA ATA Lys Thr Ile Met Pro Phe Asp Leu Ser Ile Tyr Thr Asn Asp Thr Ile 835 840 845	2544
CTA ATA GAA ATG TTT AAT AAA TAT AAT AGC GAA ATT TTA AAT AAT ATT Leu Ile Glu Met Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile 850 855 860	2592
ATC TTA AAT TTA AGA TAT AAG GAT AAT AAT TTA ATA GAT TTA TCA GGA Ile Leu Asn Leu Arg Tyr Lys Asp Asn Asn Leu Ile Asp Leu Ser Gly 865 870 875 880	2640
TAT GGG GCA AAG GTA GAG GTA TAT GAT GGA GTC GAG CTT AAT GAT AAA Tyr Gly Ala Lys Val Glu Val Tyr Asp Gly Val Glu Leu Asn Asp Lys 885 890 895	2688
AAT CAA TTT AAA TTA ACT AGT TCA GCA AAT AGT AAG ATT AGA GTG ACT Asn Gln Phe Lys Leu Thr Ser Ser Ala Asn Ser Lys Ile Arg Val Thr 900 905 910	2736
CAA AAT CAG AAT ATC ATA TTT AAT AGT GTG TTC CTT GAT TTT AGC GTT Gln Asn Gln Asn Ile Ile Phe Asn Ser Val Phe Leu Asp Phe Ser Val 915 920 925	2784
AGC TTT TGG ATA AGA ATA CCT AAA TAT AAG AAT GAT GGT ATA CAA AAT Ser Phe Trp Ile Arg Ile Pro Lys Tyr Lys Asn Asp Gly Ile Gln Asn 930 935 940	2832
TAT ATT CAT AAT GAA TAT ACA ATA ATT AAT TGT ATG AAA AAT AAT TCG Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser 945 950 955 960	2880
GGC TGG AAA ATA TCT ATT AGG GGT AAT AGG ATA ATA TGG ACT TTA ATT Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile 965 970 975	2928

GAT ATA AAT GGA AAA ACC AAA TCG GTA TTT TTT GAA TAT AAC ATA AGA Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg 980 985 990	2976
GAA GAT ATA TCA GAG TAT ATA AAT AGA TGG TTT TTT GTA ACT ATT ACT Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr 995 1000 1005	3024
AAT AAT TTG AAT AAC GCT AAA ATT TAT ATT AAT GGT AAG CTA GAA TCA Asn Asn Leu Asn Asn Ala Lys Ile Tyr Ile Asn Gly Lys Leu Glu Ser 1010 1015 1020	3072
AAT ACA GAT ATT AAA GAT ATA AGA GAA GTT ATT GCT AAT GGT GAA ATA Asn Thr Asp Ile Lys Asp Ile Arg Glu Val Ile Ala Asn Gly Glu Ile 1025 1030 1035 1040	3120
ATA TTT AAA TTA GAT GGT GAT ATA GAT AGA ACA CAA TTT ATT TGG ATG Ile Phe Lys Leu Asp Gly Asp Ile Asp Arg Thr Gln Phe Ile Trp Met 1045 1050 1055	3168
AAA TAT TTC AGT ATT TTT AAT ACG GAA TTA AGT CAA TCA AAT ATT GAA Lys Tyr Phe Ser Ile Phe Asn Thr Glu Leu Ser Gln Ser Asn Ile Glu 1060 1065 1070	3216
GAA AGA TAT AAA ATT CAA TCA TAT AGC GAA TAT TTA AAA GAT TTT TGG Glu Arg Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp 1075 1080 1085	3264
GGA AAT CCT TTA ATG TAC AAT AAA GAA TAT TAT ATG TTT AAT GCG GGG Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly 1090 1095 1100	3312
AAT AAA AAT TCA TAT ATT AAA CTA AAG AAA GAT TCA CCT GTA GGT GAA Asn Lys Asn Ser Tyr Ile Lys Leu Lys Lys Asp Ser Pro Val Gly Glu 1105 1110 1115 1120	3360
ATT TTA ACA CGT AGC AAA TAT AAT CAA AAT TCT AAA TAT ATA AAT TAT Ile Leu Thr Arg Ser Lys Tyr Asn Gln Asn Ser Lys Tyr Ile Asn Tyr 1125 1130 1135	3408
AGA GAT TTA TAT ATT GGA GAA AAA TTT ATT ATA AGA AGA AAG TCA AAT Arg Asp Leu Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Lys Ser Asn 1140 1145 1150	3456
TCT CAA TCT ATA AAT GAT GAT ATA GTT AGA AAA GAA GAT TAT ATA TAT Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr 1155 1160 1165	3504
CTA GAT TTT TTT AAT TTA AAT CAA GAG TGG AGA GTA TAT ACC TAT AAA Leu Asp Phe Phe Asn Leu Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys 1170 1175 1180	3552
TAT TTT AAG AAA GAG GAA GAA AAA TTG TTT TTA GCT CCT ATA AGT GAT Tyr Phe Lys Lys Glu Glu Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp 1185 1190 1195 1200	3600
TCT GAT GAG TTT TAC AAT ACT ATA CAA ATA AAA GAA TAT GAT GAA CAG Ser Asp Glu Phe Tyr Asn Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln 1205 1210 1215	3648

CCA ACA TAT AGT TGT CAG TTG CTT TTT AAA AAA GAT GAA GAA AGT ACT Pro Thr Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr 1220 1225 1230	3696
GAT GAG ATA GGA TTG ATT GGT ATT CAT CGT TTC TAC GAA TCT GGA ATT Asp Glu Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Ile 1235 1240 1245	3744
GTA TTT GAA GAG TAT AAA GAT TAT TTT TGT ATA AGT AAA TGG TAC TTA Val Phe Glu Glu Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu 1250 1255 1260	3792
AAA GAG GTA AAA AGG AAA CCA TAT AAT TTA AAA TTG GGA TGT AAT TGG Lys Glu Val Lys Arg Lys Pro Tyr Asn Leu Lys Leu Gly Cys Asn Trp 1265 1270 1275 1280	3840
CAG TTT ATT CCT AAA GAT GAA GGG TGG ACT GAA TAA Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu 1285 1290	3876

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1291 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Met Pro Val Thr Ile Asn Asn Phe Asn Tyr Asn Asp Pro Ile Asp Asn 1 5 10 15
Asn Asn Ile Ile Met Met Glu Pro Pro Phe Ala Arg Gly Thr Gly Arg 20 25 30
Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Ile Pro Glu 35 40 45
Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser Ser Gly 50 55 60
Ile Phe Asn Arg Asp Val Cys Glu Tyr Tyr Asp Pro Asp Tyr Leu Asn 65 70 75 80
Thr Asn Asp Lys Lys Asn Ile Phe Leu Gln Thr Met Ile Lys Leu Phe 85 90 95
Asn Arg Ile Lys Ser Lys Pro Leu Gly Glu Lys Leu Leu Glu Met Ile 100 105 110
Ile Asn Gly Ile Pro Tyr Leu Gly Asp Arg Arg Val Pro Leu Glu Glu 115 120 125
Phe Asn Thr Asn Ile Ala Ser Val Thr Val Asn Lys Leu Ile Ser Asn 130 135 140
Pro Gly Glu Val Glu Arg Lys Lys Gly Ile Phe Ala Asn Leu Ile Ile 145 150 155 160

Phe Gly Pro Gly Pro Val Leu Asn Glu Asn Glu Thr Ile Asp Ile Gly  
 165 170 175  
 Ile Gln Asn His Phe Ala Ser Arg Glu Gly Phe Gly Gly Ile Met Gln  
 180 185 190  
 Met Lys Phe Cys Pro Glu Tyr Val Ser Val Phe Asn Asn Val Gln Glu  
 195 200 205  
 Asn Lys Gly Ala Ser Ile Phe Asn Arg Arg Gly Tyr Phe Ser Asp Pro  
 210 215 220  
 Ala Leu Ile Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr  
 225 230 235 240  
 Gly Ile Lys Val Asp Asp Leu Pro Ile Val Pro Asn Glu Lys Lys Phe  
 245 250 255  
 Phe Met Gln Ser Thr Asp Ala Ile Gln Ala Glu Glu Leu Tyr Thr Phe  
 260 265 270  
 Gly Gly Gln Asp Pro Ser Ile Ile Thr Pro Ser Thr Asp Lys Ser Ile  
 275 280 285  
 Tyr Asp Lys Val Leu Gln Asn Phe Arg Gly Ile Val Asp Arg Leu Asn  
 290 295 300  
 Lys Val Leu Val Cys Ile Ser Asp Pro Asn Ile Asn Ile Asn Ile Tyr  
 305 310 315 320  
 Lys Asn Lys Phe Lys Asp Lys Tyr Lys Phe Val Glu Asp Ser Glu Gly  
 325 330 335  
 Lys Tyr Ser Ile Asp Val Glu Ser Phe Asp Lys Leu Tyr Lys Ser Leu  
 340 345 350  
 Met Phe Gly Phe Thr Glu Thr Asn Ile Ala Glu Asn Tyr Lys Ile Lys  
 355 360 365  
 Thr Arg Ala Ser Tyr Phe Ser Asp Ser Leu Pro Pro Val Lys Ile Lys  
 370 375 380  
 Asn Leu Leu Asp Asn Glu Ile Tyr Thr Ile Glu Glu Gly Phe Asn Ile  
 385 390 395 400  
 Ser Asp Lys Asp Met Glu Lys Glu Tyr Arg Gly Gln Asn Lys Ala Ile  
 405 410 415  
 Asn Lys Gln Ala Tyr Glu Glu Ile Ser Lys Glu His Leu Ala Val Tyr  
 420 425 430  
 Lys Ile Gln Met Cys Lys Ser Val Lys Ala Pro Gly Ile Cys Ile Asp  
 435 440 445  
 Val Asp Asn Glu Asp Leu Phe Phe Ile Ala Asp Lys Asn Ser Phe Ser  
 450 455 460  
 Asp Asp Leu Ser Lys Asn Glu Arg Ile Glu Tyr Asn Thr Gln Ser Asn  
 465 470 475 480  
 Tyr Ile Glu Asn Asp Phe Pro Ile Asn Glu Leu Ile Leu Asp Thr Asp  
 485 490 495

Leu Ile Ser Lys Ile Glu Leu Pro Ser Glu Asn Thr Glu Ser Leu Thr  
 500 505 510  
 Asp Phe Asn Val Asp Val Pro Val Tyr Glu Lys Gln Pro Ala Ile Lys  
 515 520 525  
 Lys Ile Phe Thr Asp Glu Asn Thr Ile Phe Gln Tyr Leu Tyr Ser Gln  
 530 535 540  
 Thr Phe Leu Leu Asp Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp  
 545 550 555 560  
 Asp Ala Leu Leu Phe Ser Asn Lys Val Tyr Ser Phe Phe Ser Met Asp  
 565 570 575  
 Tyr Ile Lys Thr Ala Asn Lys Val Val Glu Ala Gly Leu Phe Ala Gly  
 580 585 590  
 Trp Val Lys Gln Ile Val Asn Asp Phe Val Ile Glu Ala Asn Lys Ser  
 595 600 605  
 Asn Thr Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile  
 610 615 620  
 Gly Leu Ala Leu Asn Val Gly Asn Glu Thr Ala Lys Gly Asn Phe Glu  
 625 630 635 640  
 Asn Ala Phe Glu Ile Ala Gly Ala Ser Ile Leu Leu Glu Phe Ile Pro  
 645 650 655  
 Glu Leu Leu Ile Pro Val Val Gly Ala Phe Leu Leu Glu Ser Tyr Ile  
 660 665 670  
 Asp Asn Lys Asn Lys Ile Ile Lys Thr Ile Asp Asn Ala Leu Thr Lys  
 675 680 685  
 Arg Asn Glu Lys Trp Ser Asp Met Tyr Gly Leu Ile Val Ala Gln Trp  
 690 695 700  
 Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile Lys Glu Gly Met Tyr  
 705 710 715 720  
 Lys Ala Leu Asn Tyr Gln Ala Gln Ala Leu Glu Glu Ile Ile Lys Tyr  
 725 730 735  
 Arg Tyr Asn Ile Tyr Ser Glu Lys Glu Lys Ser Asn Ile Asn Ile Asp  
 740 745 750  
 Phe Asn Asp Ile Asn Ser Lys Leu Asn Glu Gly Ile Asn Gln Ala Ile  
 755 760 765  
 Asp Asn Ile Asn Asn Phe Ile Asn Gly Cys Ser Val Ser Tyr Leu Met  
 770 775 780  
 Lys Lys Met Ile Pro Leu Ala Val Glu Lys Leu Leu Asp Phe Asp Asn  
 785 790 795 800  
 Thr Leu Lys Lys Asn Leu Leu Asn Tyr Ile Asp Glu Asn Lys Leu Tyr  
 805 810 815  
 Leu Ile Gly Ser Ala Glu Tyr Glu Lys Ser Lys Val Asn Lys Tyr Leu  
 820 825 830

Lys Thr Ile Met Pro Phe Asp Leu Ser Ile Tyr Thr Asn Asp Thr Ile  
 835 840 845  
 Leu Ile Glu Met Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile  
 850 855 860  
 Ile Leu Asn Leu Arg Tyr Lys Asp Asn Asn Leu Ile Asp Leu Ser Gly  
 865 870 875 880  
 Tyr Gly Ala Lys Val Glu Val Tyr Asp Gly Val Glu Leu Asn Asp Lys  
 885 890 895  
 Asn Gln Phe Lys Leu Thr Ser Ser Ala Asn Ser Lys Ile Arg Val Thr  
 900 905 910  
 Gln Asn Gln Asn Ile Ile Phe Asn Ser Val Phe Leu Asp Phe Ser Val  
 915 920 925  
 Ser Phe Trp Ile Arg Ile Pro Lys Tyr Lys Asn Asp Gly Ile Gln Asn  
 930 935 940  
 Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser  
 945 950 955 960  
 Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile  
 965 970 975  
 Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg  
 980 985 990  
 Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr  
 995 1000 1005  
 Asn Asn Leu Asn Asn Ala Lys Ile Tyr Ile Asn Gly Lys Leu Glu Ser  
 1010 1015 1020  
 Asn Thr Asp Ile Lys Asp Ile Arg Glu Val Ile Ala Asn Gly Glu Ile  
 1025 1030 1035 1040  
 Ile Phe Lys Leu Asp Gly Asp Ile Asp Arg Thr Gln Phe Ile Trp Met  
 1045 1050 1055  
 Lys Tyr Phe Ser Ile Phe Asn Thr Glu Leu Ser Gln Ser Asn Ile Glu  
 1060 1065 1070  
 Glu Arg Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp  
 1075 1080 1085  
 Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly  
 1090 1095 1100  
 Asn Lys Asn Ser Tyr Ile Lys Leu Lys Lys Asp Ser Pro Val Gly Glu  
 1105 1110 1115 1120  
 Ile Leu Thr Arg Ser Lys Tyr Asn Gln Asn Ser Lys Tyr Ile Asn Tyr  
 1125 1130 1135  
 Arg Asp Leu Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Lys Ser Asn  
 1140 1145 1150  
 Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr  
 1155 1160 1165

Leu Asp Phe Phe Asn Leu Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys  
 1170 1175 1180  
 Tyr Phe Lys Lys Glu Glu Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp  
 1185 1190 1195 1200  
 Ser Asp Glu Phe Tyr Asn Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln  
 1205 1210 1215  
 Pro Thr Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr  
 1220 1225 1230  
 Asp Glu Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Ile  
 1235 1240 1245  
 Val Phe Glu Glu Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu  
 1250 1255 1260  
 Lys Glu Val Lys Arg Lys Pro Tyr Asn Leu Lys Leu Gly Cys Asn Trp  
 1265 1270 1275 1280  
 Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu  
 1285 1290

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1526 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "DNA"

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 108..1523

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATAACC ATG GGC CAT	116
	Met Gly His
	1
CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT	164
His His His His His His His His His Ser Ser Gly His Ile Glu Gly	
5 10 15	
CGT CAT ATG GCT AGC ATG GCT GAT ACA ATA CTA ATA GAA ATG TTT AAT	212
Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Glu Met Phe Asn	
20 25 30 35	
AAA TAT AAT AGC GAA ATT TTA AAT AAT ATT ATC TTA AAT TTA AGA TAT	260
Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile Ile Leu Asn Leu Arg Tyr	
40 45 50	
AGA GAT AAT AAT TTA ATA GAT TTA TCA GGA TAT GGA GCA AAG GTA GAG	308
Arg Asp Asn Asn Leu Ile Asp Leu Ser Gly Tyr Gly Ala Lys Val Glu	
55 60 65	

GTA TAT GAT GGG GTC AAG CTT AAT GAT AAA AAT CAA TTT AAA TTA ACT Val Tyr Asp Gly Val Lys Leu Asn Asp Lys Asn Gln Phe Lys Leu Thr 70 75 80	356
AGT TCA GCA GAT AGT AAG ATT AGA GTC ACT CAA AAT CAG AAT ATT ATA Ser Ser Ala Asp Ser Lys Ile Arg Val Thr Gln Asn Gln Asn Ile Ile 85 90 95	404
TTT AAT AGT ATG TTC CTT GAT TTT AGC GTT AGC TTT TGG ATA AGG ATA Phe Asn Ser Met Phe Leu Asp Phe Ser Val Ser Phe Trp Ile Arg Ile 100 105 110 115	452
CCT AAA TAT AGG AAT GAT GAT ATA CAA AAT TAT ATT CAT AAT GAA TAT Pro Lys Tyr Arg Asn Asp Asp Ile Gln Asn Tyr Ile His Asn Glu Tyr 120 125 130	500
ACG ATA ATT AAT TGT ATG AAA AAT AAT TCA GGC TGG AAA ATA TCT ATT Thr Ile Ile Asn Cys Met Lys Asn Asn Ser Gly Trp Lys Ile Ser Ile 135 140 145	548
AGG GGT AAT AGG ATA ATA TGG ACC TTA ATT GAT ATA AAT GGA AAA ACC Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile Asp Ile Asn Gly Lys Thr 150 155 160	596
AAA TCA GTA TTT TTT GAA TAT AAC ATA AGA GAA GAT ATA TCA GAG TAT Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg Glu Asp Ile Ser Glu Tyr 165 170 175	644
ATA AAT AGA TGG TTT TTT GTA ACT ATT ACT AAT AAT TTG GAT AAT GCT Ile Asn Arg Trp Phe Phe Val Thr Ile Thr Asn Asn Leu Asp Asn Ala 180 185 190 195	692
AAA ATT TAT ATT AAT GGC ACG TTA GAA TCA AAT ATG GAT ATT AAA GAT Lys Ile Tyr Ile Asn Gly Thr Leu Glu Ser Asn Met Asp Ile Lys Asp 200 205 210	740
ATA GGA GAA GTT ATT GTT AAT GGT GAA ATA ACA TTT AAA TTA GAT GGT Ile Gly Glu Val Ile Val Asn Gly Glu Ile Thr Phe Lys Leu Asp Gly 215 220 225	788
GAT GTA GAT AGA ACA CAA TTT ATT TGG ATG AAA TAT TTT AGT ATT TTT Asp Val Asp Arg Thr Gln Phe Ile Trp Met Lys Tyr Phe Ser Ile Phe 230 235 240	836
AAT ACG CAA TTA AAT CAA TCA AAT ATT AAA GAG ATA TAT AAA ATT CAA Asn Thr Gln Leu Asn Gln Ser Asn Ile Lys Glu Ile Tyr Lys Ile Gln 245 250 255	884
TCA TAT AGC GAA TAC TTA AAA GAT TTT TGG GGA AAT CCT TTA ATG TAT Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp Gly Asn Pro Leu Met Tyr 260 265 270 275	932
AAT AAA GAA TAT TAT ATG TTT AAT GCG GGG AAT AAA AAT TCA TAT ATT Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly Asn Lys Asn Ser Tyr Ile 280 285 290	980
AAA CTA GTG AAA GAT TCA TCT GTA GGT GAA ATA TTA ATA CGT AGC AAA Lys Leu Val Lys Asp Ser Ser Val Gly Glu Ile Leu Ile Arg Ser Lys 295 300 305	1028



TAT AAT CAG AAT TCC AAT TAT ATA AAT TAT AGA AAT TTA TAT ATT GGA Tyr Asn Gln Asn Ser Asn Tyr Ile Asn Tyr Arg Asn Leu Tyr Ile Gly 310 315 320	1076
GAA AAA TTT ATT ATA AGA AGA GAG TCA AAT TCT CAA TCT ATA AAT GAT Glu Lys Phe Ile Ile Arg Arg Glu Ser Asn Ser Gln Ser Ile Asn Asp 325 330 335	1124
GAT ATA GTT AGA AAA GAA GAT TAT ATA CAT CTA GAT TTG GTA CTT CAC Asp Ile Val Arg Lys Glu Asp Tyr Ile His Leu Asp Leu Val Leu His 340 345 350 355	1172
CAT GAA GAG TGG AGA GTA TAT GCC TAT AAA TAT TTT AAG GAA CAG GAA His Glu Glu Trp Arg Val Tyr Ala Tyr Lys Tyr Phe Lys Glu Gln Glu 360 365 370	1220
GAA AAA TTG TTT TTA TCT ATT ATA AGT GAT TCT AAT GAA TTT TAT AAG Glu Lys Leu Phe Leu Ser Ile Ile Ser Asp Ser Asn Glu Phe Tyr Lys 375 380 385	1268
ACT ATA GAA ATA AAA GAA TAT GAT GAA CAG CCA TCA TAT AGT TGT CAG Thr Ile Glu Ile Lys Glu Tyr Asp Glu Gln Pro Ser Tyr Ser Cys Gln 390 395 400	1316
TTG CTT TTT AAA AAA GAT GAA GAA AGT ACT GAT GAT ATA GGA TTG ATT Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr Asp Asp Ile Gly Leu Ile 405 410 415	1364
GGT ATT CAT CGT TTC TAC GAA TCT GGA GTT TTA CGT AAA AAG TAT AAA Gly Ile His Arg Phe Tyr Glu Ser Gly Val Leu Arg Lys Lys Tyr Lys 420 425 430 435	1412
GAT TAT TTT TGT ATA AGT AAA TGG TAC TTA AAA GAG GTA AAA AGG AAA Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu Lys Glu Val Lys Arg Lys 440 445 450	1460
CCA TAT AAG TCA AAT TTG GGA TGT AAT TGG CAG TTT ATT CCT AAA GAT Pro Tyr Lys Ser Asn Leu Gly Cys Asn Trp Gln Phe Ile Pro Lys Asp 455 460 465	1508
GAA GGG TGG ACT GAA TAA Glu Gly Trp Thr Glu 470	1526

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 472 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Met Gly His His His His His His His His His His Ser Ser Gly His 1 5 10 15
Ile Glu Gly Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Glu 20 25 30

Met Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile Ile Leu Asn  
 35 40 45  
 Leu Arg Tyr Arg Asp Asn Asn Leu Ile Asp Leu Ser Gly Tyr Gly Ala  
 50 55 60  
 Lys Val Glu Val Tyr Asp Gly Val Lys Leu Asn Asp Lys Asn Gln Phe  
 65 70 75 80  
 Lys Leu Thr Ser Ser Ala Asp Ser Lys Ile Arg Val Thr Gln Asn Gln  
 85 90 95  
 Asn Ile Ile Phe Asn Ser Met Phe Leu Asp Phe Ser Val Ser Phe Trp  
 100 105 110  
 Ile Arg Ile Pro Lys Tyr Arg Asn Asp Asp Ile Gln Asn Tyr Ile His  
 115 120 125  
 Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser Gly Trp Lys  
 130 135 140  
 Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile Asp Ile Asn  
 145 150 155 160  
 Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg Glu Asp Ile  
 165 170 175  
 Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr Asn Asn Leu  
 180 185 190  
 Asp Asn Ala Lys Ile Tyr Ile Asn Gly Thr Leu Glu Ser Asn Met Asp  
 195 200 205  
 Ile Lys Asp Ile Gly Glu Val Ile Val Asn Gly Glu Ile Thr Phe Lys  
 210 215 220  
 Leu Asp Gly Asp Val Asp Arg Thr Gln Phe Ile Trp Met Lys Tyr Phe  
 225 230 235 240  
 Ser Ile Phe Asn Thr Gln Leu Asn Gln Ser Asn Ile Lys Glu Ile Tyr  
 245 250 255  
 Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp Gly Asn Pro  
 260 265 270  
 Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly Asn Lys Asn  
 275 280 285  
 Ser Tyr Ile Lys Leu Val Lys Asp Ser Ser Val Gly Glu Ile Leu Ile  
 290 295 300  
 Arg Ser Lys Tyr Asn Gln Asn Ser Asn Tyr Ile Asn Tyr Arg Asn Leu  
 305 310 315 320  
 Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Glu Ser Asn Ser Gln Ser  
 325 330 335  
 Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile His Leu Asp Leu  
 340 345 350  
 Val Leu His His Glu Glu Trp Arg Val Tyr Ala Tyr Lys Tyr Phe Lys  
 355 360 365

Glu Gln Glu Glu Lys Leu Phe Leu Ser Ile Ile Ser Asp Ser Asn Glu  
 370 375 380  
 Phe Tyr Lys Thr Ile Glu Ile Lys Glu Tyr Asp Glu Gln Pro Ser Tyr  
 385 390 395 400  
 Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr Asp Asp Ile  
 405 410 415  
 Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Val Leu Arg Lys  
 420 425 430  
 Lys Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu Lys Glu Val  
 435 440 445  
 Lys Arg Lys Pro Tyr Lys Ser Asn Leu Gly Cys Asn Trp Gln Phe Ile  
 450 455 460  
 Pro Lys Asp Glu Gly Trp Thr Glu  
 465 470

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1547 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 108..1523

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
TTCCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATAACC ATG GGC CAT	116
	Met Gly His
	1
CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT	164
His His His His His His His His His Ser Ser Gly His Ile Glu Gly	
5 10 15	
CGT CAT ATG GCT AGC ATG GCT GAT ACA ATA CTA ATA GAA ATG TTT AAT	212
Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Glu Met Phe Asn	
20 25 30 35	
AAA TAT AAT AGC GAA ATT TTA AAT AAT ATT ATC TTA AAT TTA AGA TAT	260
Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile Ile Leu Asn Leu Arg Tyr	
40 45 50	
AAG GAT AAT AAT TTA ATA GAT TTA TCA GGA TAT GGG GCA AAG GTA GAG	308
Lys Asp Asn Asn Leu Ile Asp Leu Ser Gly Tyr Gly Ala Lys Val Glu	
55 60 65	
GTA TAT GAT GGA GTC GAG CTT AAT GAT AAA AAT CAA TTT AAA TTA ACT	356
Val Tyr Asp Gly Val Glu Leu Asn Asp Lys Asn Gln Phe Lys Leu Thr	
70 75 80	

AGT TCA GCA AAT AGT AAG ATT AGA GTG ACT CAA AAT CAG AAT ATC ATA	404
Ser Ser Ala Asn Ser Lys Ile Arg Val Thr Gln Asn Gln Asn Ile Ile	
85 90 95	
TTT AAT AGT GTG TTC CTT GAT TTT AGC GTT AGC TTT TGG ATA AGA ATA	452
Phe Asn Ser Val Phe Leu Asp Phe Ser Val Ser Phe Trp Ile Arg Ile	
100 105 110 115	
CCT AAA TAT AAG AAT GAT GGT ATA CAA AAT TAT ATT CAT AAT GAA TAT	500
Pro Lys Tyr Lys Asn Asp Gly Ile Gln Asn Tyr Ile His Asn Glu Tyr	
120 125 130	
ACA ATA ATT AAT TGT ATG AAA AAT AAT TCG GGC TGG AAA ATA TCT ATT	548
Thr Ile Ile Asn Cys Met Lys Asn Asn Ser Gly Trp Lys Ile Ser Ile	
135 140 145	
AGG GGT AAT AGG ATA ATA TGG ACT TTA ATT GAT ATA AAT GGA AAA ACC	596
Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile Asp Ile Asn Gly Lys Thr	
150 155 160	
AAA TCG GTA TTT TTT GAA TAT AAC ATA AGA GAA GAT ATA TCA GAG TAT	644
Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg Glu Asp Ile Ser Glu Tyr	
165 170 175	
ATA AAT AGA TGG TTT TTT GTA ACT ATT ACT AAT AAT TTG AAT AAC GCT	692
Ile Asn Arg Trp Phe Phe Val Thr Ile Thr Asn Asn Leu Asn Asn Ala	
180 185 190 195	
AAA ATT TAT ATT AAT GGT AAG CTA GAA TCA AAT ACA GAT ATT AAA GAT	740
Lys Ile Tyr Ile Asn Gly Lys Leu Glu Ser Asn Thr Asp Ile Lys Asp	
200 205 210	
ATA AGA GAA GTT ATT GCT AAT GGT GAA ATA ATA TTT AAA TTA GAT GGT	788
Ile Arg Glu Val Ile Ala Asn Gly Glu Ile Ile Phe Lys Leu Asp Gly	
215 220 225	
GAT ATA GAT AGA ACA CAA TTT ATT TGG ATG AAA TAT TTC AGT ATT TTT	836
Asp Ile Asp Arg Thr Gln Phe Ile Trp Met Lys Tyr Phe Ser Ile Phe	
230 235 240	
AAT ACG GAA TTA AGT CAA TCA AAT ATT GAA GAA AGA TAT AAA ATT CAA	884
Asn Thr Glu Leu Ser Gln Ser Asn Ile Glu Glu Arg Tyr Lys Ile Gln	
245 250 255	
TCA TAT AGC GAA TAT TTA AAA GAT TTT TGG GGA AAT CCT TTA ATG TAC	932
Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp Gly Asn Pro Leu Met Tyr	
260 265 270 275	
AAT AAA GAA TAT TAT ATG TTT AAT GCG GGG AAT AAA AAT TCA TAT ATT	980
Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly Asn Lys Asn Ser Tyr Ile	
280 285 290	
AAA CTA AAG AAA GAT TCA CCT GTA GGT GAA ATT TTA ACA CGT AGC AAA	1028
Lys Leu Lys Lys Asp Ser Pro Val Gly Glu Ile Leu Thr Arg Ser Lys	
295 300 305	
TAT AAT CAA AAT TCT AAA TAT ATA AAT TAT AGA GAT TTA TAT ATT GGA	1076
Tyr Asn Gln Asn Ser Lys Tyr Ile Asn Tyr Arg Asp Leu Tyr Ile Gly	
310 315 320	

GAA AAA TTT ATT ATA AGA AGA AAG TCA AAT TCT CAA TCT ATA AAT GAT Glu Lys Phe Ile Ile Arg Arg Lys Ser Asn Ser Gln Ser Ile Asn Asp 325 330 335	1124
GAT ATA GTT AGA AAA GAA GAT TAT ATA TAT CTA GAT TTT TTT AAT TTA Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr Leu Asp Phe Phe Asn Leu 340 345 350 355	1172
AAT CAA GAG TGG AGA GTA TAT ACC TAT AAA TAT TTT AAG AAA GAG GAA Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys Tyr Phe Lys Lys Glu Glu 360 365 370	1220
GAA AAA TTG TTT TTA GCT CCT ATA AGT GAT TCT GAT GAG TTT TAC AAT Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp Ser Asp Glu Phe Tyr Asn 375 380 385	1268
ACT ATA CAA ATA AAA GAA TAT GAT GAA CAG CCA ACA TAT AGT TGT CAG Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln Pro Thr Tyr Ser Cys Gln 390 395 400	1316
TTG CTT TTT AAA AAA GAT GAA GAA AGT ACT GAT GAG ATA GGA TTG ATT Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr Asp Glu Ile Gly Leu Ile 405 410 415	1364
GGT ATT CAT CGT TTC TAC GAA TCT GGA ATT GTA TTT GAA GAG TAT AAA Gly Ile His Arg Phe Tyr Glu Ser Gly Ile Val Phe Glu Glu Tyr Lys 420 425 430 435	1412
GAT TAT TTT TGT ATA AGT AAA TGG TAC TTA AAA GAG GTA AAA AGG AAA Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu Lys Glu Val Lys Arg Lys 440 445 450	1460
CCA TAT AAT TTA AAA TTG GGA TGT AAT TGG CAG TTT ATT CCT AAA GAT Pro Tyr Asn Leu Lys Leu Gly Cys Asn Trp Gln Phe Ile Pro Lys Asp 455 460 465	1508
GAA GGG TGG ACT GAA TAAAAGCTTG CGGCCGCACT CGAG Glu Gly Trp Thr Glu 470	1547

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 472 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Met Gly His His His His His His His His His His Ser Ser Gly His 1 5 10 15
Ile Glu Gly Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Glu 20 25 30
Met Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile Ile Leu Asn 35 40 45
Leu Arg Tyr Lys Asp Asn Asn Leu Ile Asp Leu Ser Gly Tyr Gly Ala 50 55 60

Lys Val Glu Val Tyr Asp Gly Val Glu Leu Asn Asp Lys Asn Gln Phe  
 65 70 75 80  
 Lys Leu Thr Ser Ser Ala Asn Ser Lys Ile Arg Val Thr Gln Asn Gln  
 85 90 95  
 Asn Ile Ile Phe Asn Ser Val Phe Leu Asp Phe Ser Val Ser Phe Trp  
 100 105 110  
 Ile Arg Ile Pro Lys Tyr Lys Asn Asp Gly Ile Gln Asn Tyr Ile His  
 115 120 125  
 Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser Gly Trp Lys  
 130 135 140  
 Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile Asp Ile Asn  
 145 150 155 160  
 Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg Glu Asp Ile  
 165 170 175  
 Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr Asn Asn Leu  
 180 185 190  
 Asn Asn Ala Lys Ile Tyr Ile Asn Gly Lys Leu Glu Ser Asn Thr Asp  
 195 200 205  
 Ile Lys Asp Ile Arg Glu Val Ile Ala Asn Gly Glu Ile Ile Phe Lys  
 210 215 220  
 Leu Asp Gly Asp Ile Asp Arg Thr Gln Phe Ile Trp Met Lys Tyr Phe  
 225 230 235 240  
 Ser Ile Phe Asn Thr Glu Leu Ser Gln Ser Asn Ile Glu Glu Arg Tyr  
 245 250 255  
 Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp Gly Asn Pro  
 260 265 270  
 Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly Asn Lys Asn  
 275 280 285  
 Ser Tyr Ile Lys Leu Lys Lys Asp Ser Pro Val Gly Glu Ile Leu Thr  
 290 295 300  
 Arg Ser Lys Tyr Asn Gln Asn Ser Lys Tyr Ile Asn Tyr Arg Asp Leu  
 305 310 315 320  
 Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Lys Ser Asn Ser Gln Ser  
 325 330 335  
 Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr Leu Asp Phe  
 340 345 350  
 Phe Asn Leu Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys Tyr Phe Lys  
 355 360 365  
 Lys Glu Glu Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp Ser Asp Glu  
 370 375 380  
 Phe Tyr Asn Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln Pro Thr Tyr  
 385 390 395 400

Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr Asp Glu Ile  
405 410 415

Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Ile Val Phe Glu  
420 425 430

Glu Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu Lys Glu Val  
435 440 445

Lys Arg Lys Pro Tyr Asn Leu Lys Leu Gly Cys Asn Trp Gln Phe Ile  
450 455 460

Pro Lys Asp Glu Gly Trp Thr Glu  
465 470

(2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 31 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CGCCATGGCT GATACAATAC TAATAGAAAT G

31

(2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GCAAGCTTTT ATTCAGTCCA CCCTTCATC

29

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 3753 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 1..3750

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

ATG CCA ACA ATT AAT AGT TTT AAT TAT AAT GAT CCT GTT AAT AAT AGA	48
Met Pro Thr Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asn Arg	
1 5 10 15	
ACA ATT TTA TAT ATT AAA CCA GGC GGT TGT CAA CAA TTT TAT AAA TCA	96
Thr Ile Leu Tyr Ile Lys Pro Gly Gly Cys Gln Gln Phe Tyr Lys Ser	
20 25 30	
TTT AAT ATT ATG AAA AAT ATT TGG ATA ATT CCA GAG AGA AAT GTA ATT	144
Phe Asn Ile Met Lys Asn Ile Trp Ile Ile Pro Glu Arg Asn Val Ile	
35 40 45	
GGT ACA ATT CCC CAA GAT TTT CTT CCG CCT ACT TCA TTG AAA AAT GGA	192
Gly Thr Ile Pro Gln Asp Phe Leu Pro Pro Thr Ser Leu Lys Asn Gly	
50 55 60	
GAT AGT AGT TAT TAT GAC CCT AAT TAT TTA CAA AGT GAT CAA GAA AAG	240
Asp Ser Ser Tyr Tyr Asp Pro Asn Tyr Leu Gln Ser Asp Gln Glu Lys	
65 70 75 80	
GAT AAA TTT TTA AAA ATA GTC ACA AAA ATA TTT AAT AGA ATA AAT GAT	288
Asp Lys Phe Leu Lys Ile Val Thr Lys Ile Phe Asn Arg Ile Asn Asp	
85 90 95	
AAT CTT TCA GGA AGG ATT TTA TTA GAA GAA CTG TCA AAA GCT AAT CCA	336
Asn Leu Ser Gly Arg Ile Leu Leu Glu Glu Leu Ser Lys Ala Asn Pro	
100 105 110	
TAT TTA GGA AAT GAT AAT ACT CCA GAT GGT GAC TTC ATT ATT AAT GAT	384
Tyr Leu Gly Asn Asp Asn Thr Pro Asp Gly Asp Phe Ile Ile Asn Asp	
115 120 125	
GCA TCA GCA GTT CCA ATT CAA TTC TCA AAT GGT AGC CAA AGC ATA CTA	432
Ala Ser Ala Val Pro Ile Gln Phe Ser Asn Gly Ser Gln Ser Ile Leu	
130 135 140	
TTA CCT AAT GTT ATT ATA ATG GGA GCA GAG CCT GAT TTA TTT GAA ACT	480
Leu Pro Asn Val Ile Ile Met Gly Ala Glu Pro Asp Leu Phe Glu Thr	
145 150 155 160	
AAC AGT TCC AAT ATT TCT CTA AGA AAT AAT TAT ATG CCA AGC AAT CAC	528
Asn Ser Ser Asn Ile Ser Leu Arg Asn Asn Tyr Met Pro Ser Asn His	
165 170 175	
GGT TTT GGA TCA ATA GCT ATA GTA ACA TTC TCA CCT GAA TAT TCT TTT	576
Gly Phe Gly Ser Ile Ala Ile Val Thr Phe Ser Pro Glu Tyr Ser Phe	
180 185 190	
AGA TTT AAA GAT AAT AGT ATG AAT GAA TTT ATT CAA GAT CCT GCT CTT	624
Arg Phe Lys Asp Asn Ser Met Asn Glu Phe Ile Gln Asp Pro Ala Leu	
195 200 205	
ACA TTA ATG CAT GAA TTA ATA CAT TCA TTA CAT GGA CTA TAT GGG GCT	672
Thr Leu Met His Glu Leu Ile His Ser Leu His Gly Leu Tyr Gly Ala	
210 215 220	
AAA GGG ATT ACT ACA AAG TAT ACT ATA ACA CAA AAA CAA AAT CCC CTA	720
Lys Gly Ile Thr Thr Lys Tyr Thr Ile Thr Gln Lys Gln Asn Pro Leu	
225 230 235 240	



ATA ACA AAT ATA AGA GGT ACA AAT ATT GAA GAA TTC TTA ACT TTT GGA Ile Thr Asn Ile Arg Gly Thr Asn Ile Glu Glu Phe Leu Thr Phe Gly 245 250 255	768
GGT ACT GAT TTA AAC ATT ATT ACT AGT GCT CAG TCC AAT GAT ATC TAT Gly Thr Asp Leu Asn Ile Ile Thr Ser Ala Gln Ser Asn Asp Ile Tyr 260 265 270	816
ACT AAT CTT CTA GCT GAT TAT AAA AAA ATA GCG TCT AAA CTT AGC AAA Thr Asn Leu Leu Ala Asp Tyr Lys Lys Ile Ala Ser Lys Leu Ser Lys 275 280 285	864
GTA CAA GTA TCT AAT CCA CTA CTT AAT CCT TAT AAA GAT GTT TTT GAA Val Gln Val Ser Asn Pro Leu Leu Asn Pro Tyr Lys Asp Val Phe Glu 290 295 300	912
GCA AAG TAT GGA TTA GAT AAA GAT GCT AGC GGA ATT TAT TCG GTA AAT Ala Lys Tyr Gly Leu Asp Lys Asp Ala Ser Gly Ile Tyr Ser Val Asn 305 310 315 320	960
ATA AAC AAA TTT AAT GAT ATT TTT AAA AAA TTA TAC AGC TTT ACG GAA Ile Asn Lys Phe Asn Asp Ile Phe Lys Lys Leu Tyr Ser Phe Thr Glu 325 330 335	1008
TTT GAT TTA GCA ACT AAA TTT CAA GTT AAA TGT AGG CAA ACT TAT ATT Phe Asp Leu Ala Thr Lys Phe Gln Val Lys Cys Arg Gln Thr Tyr Ile 340 345 350	1056
GGA CAG TAT AAA TAC TTC AAA CTT TCA AAC TTG TTA AAT GAT TCT ATT Gly Gln Tyr Lys Tyr Phe Lys Leu Ser Asn Leu Leu Asn Asp Ser Ile 355 360 365	1104
TAT AAT ATA TCA GAA GGC TAT AAT ATA AAT AAT TTA AAG GTA AAT TTT Tyr Asn Ile Ser Glu Gly Tyr Asn Ile Asn Asn Leu Lys Val Asn Phe 370 375 380	1152
AGA GGA CAG AAT GCA AAT TTA AAT CCT AGA ATT ATT ACA CCA ATT ACA Arg Gly Gln Asn Ala Asn Leu Asn Pro Arg Ile Ile Thr Pro Ile Thr 385 390 395 400	1200
GGT AGA GGA CTA GTA AAA AAA ATC ATT AGA TTT TGT AAA AAT ATT GTT Gly Arg Gly Leu Val Lys Lys Ile Ile Arg Phe Cys Lys Asn Ile Val 405 410 415	1248
TCT GTA AAA GGC ATA AGG AAA TCA ATA TGT ATC GAA ATA AAT AAT GGT Ser Val Lys Gly Ile Arg Lys Ser Ile Cys Ile Glu Ile Asn Asn Gly 420 425 430	1296
GAG TTA TTT TTT GTG GCT TCC GAG AAT AGT TAT AAT GAT GAT AAT ATA Glu Leu Phe Phe Val Ala Ser Glu Asn Ser Tyr Asn Asp Asp Asn Ile 435 440 445	1344
AAT ACT CCT AAA GAA ATT GAC GAT ACA GTA ACT TCA AAT AAT AAT TAT Asn Thr Pro Lys Glu Ile Asp Asp Thr Val Thr Ser Asn Asn Asn Tyr 450 455 460	1392
GAA AAT GAT TTA GAT CAG GTT ATT TTA AAT TTT AAT AGT GAA TCA GCA Glu Asn Asp Leu Asp Gln Val Ile Leu Asn Phe Asn Ser Glu Ser Ala 465 470 475 480	1440

CCT GGA CTT TCA GAT GAA AAA TTA AAT TTA ACT ATC CAA AAT GAT GCT Pro Gly Leu Ser Asp Glu Lys Leu Asn Leu Thr Ile Gln Asn Asp Ala 485 490 495	1488
TAT ATA CCA AAA TAT GAT TCT AAT GGA ACA AGT GAT ATA GAA CAA CAT Tyr Ile Pro Lys Tyr Asp Ser Asn Gly Thr Ser Asp Ile Glu Gln His 500 505 510	1536
GAT GTT AAT GAA CTT AAT GTA TTT TTC TAT TTA GAT GCA CAG AAA GTG Asp Val Asn Glu Leu Asn Val Phe Phe Tyr Leu Asp Ala Gln Lys Val 515 520 525	1584
CCC GAA GGT GAA AAT AAT GTC AAT CTC ACC TCT TCA ATT GAT ACA GCA Pro Glu Gly Glu Asn Asn Val Asn Leu Thr Ser Ser Ile Asp Thr Ala 530 535 540	1632
TTA TTA GAA CAA CCT AAA ATA TAT ACA TTT TTT TCA TCA GAA TTT ATT Leu Leu Glu Gln Pro Lys Ile Tyr Thr Phe Phe Ser Ser Glu Phe Ile 545 550 555 560	1680
AAT AAT GTC AAT AAA CCT GTG CAA GCA GCA TTA TTT GTA AGC TGG ATA Asn Asn Val Asn Lys Pro Val Gln Ala Ala Leu Phe Val Ser Trp Ile 565 570 575	1728
CAA CAA GTA TTA GTA GAT TTT ACT ACT GAA GCT AAC CAA AAA AGT ACT Gln Gln Val Leu Val Asp Phe Thr Thr Glu Ala Asn Gln Lys Ser Thr 580 585 590	1776
GTT GAT AAA ATT GCA GAT ATT TCT ATA GTT GTT CCA TAT ATA GGT CTT Val Asp Lys Ile Ala Asp Ile Ser Ile Val Val Pro Tyr Ile Gly Leu 595 600 605	1824
GCT TTA AAT ATA GGA AAT GAA GCA CAA AAA GGA AAT TTT AAA GAT GCA Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Phe Lys Asp Ala 610 615 620	1872
CTT GAA TTA TTA GGA GCA GGT ATT TTA TTA GAA TTT GAA CCC GAG CTT Leu Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Glu Pro Glu Leu 625 630 635 640	1920
TTA ATT CCT ACA ATT TTA GTA TTC ACG ATA AAA TCT TTT TTA GGT TCA Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser 645 650 655	1968
TCT GAT AAT AAA AAT AAA GTT ATT AAA GCA ATA AAT AAT GCA TTG AAA Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Asn Ala Leu Lys 660 665 670	2016
GAA AGA GAT GAA AAA TGG AAA GAA GTA TAT AGT TTT ATA GTA TCG AAT Glu Arg Asp Glu Lys Trp Lys Glu Val Tyr Ser Phe Ile Val Ser Asn 675 680 685	2064
TGG ATG ACT AAA ATT AAT ACA CAA TTT AAT AAA AGA AAA GAA CAA ATG Trp Met Thr Lys Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln Met 690 695 700	2112
TAT CAA GCT TTA CAA AAT CAA GTA AAT GCA CTT AAA GCA ATA ATA GAA Tyr Gln Ala Leu Gln Asn Gln Val Asn Ala Leu Lys Ala Ile Ile Glu 705 710 715 720	2160

TCT AAG TAT AAT AGT TAT ACT TTA GAA GAA AAA AAT GAG CTT ACA AAT Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Glu Lys Asn Glu Leu Thr Asn 725 730 735	2208
AAA TAT GAT ATT GAG CAA ATA GAA AAT GAA CTT AAT CAA AAG GTT TCT Lys Tyr Asp Ile Glu Gln Ile Glu Asn Glu Leu Asn Gln Lys Val Ser 740 745 750	2256
ATA GCA ATG AAT AAT ATA GAC AGG TTC TTA ACT GAA AGT TCT ATA TCT Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser 755 760 765	2304
TAT TTA ATG AAA TTA ATA AAT GAA GTA AAA ATT AAT AAA TTA AGA GAA Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu 770 775 780	2352
TAT GAT GAA AAT GTT AAA ACG TAT TTA TTA GAT TAT ATT ATA AAA CAT Tyr Asp Glu Asn Val Lys Thr Tyr Leu Leu Asp Tyr Ile Ile Lys His 785 790 795 800	2400
GGA TCA ATC TTG GGA GAG AGT CAG CAA GAA CTA AAT TCT ATG GTA ATT Gly Ser Ile Leu Gly Glu Ser Gln Gln Glu Leu Asn Ser Met Val Ile 805 810 815	2448
GAT ACC CTA AAT AAT AGT ATT CCT TTT AAG CTT TCT TCT TAT ACA GAT Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp 820 825 830	2496
GAT AAA ATT TTA ATT TCA TAT TTT AAT AAG TTC TTT AAG AGA ATT AAA Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys 835 840 845	2544
AGT AGT TCT GTT TTA AAT ATG AGA TAT AAA AAT GAT AAA TAC GTA GAT Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp 850 855 860	2592
ACT TCA GGA TAT GAT TCA AAT ATA AAT ATT AAT GGA GAT GTA TAT AAA Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys 865 870 875 880	2640
TAT CCA ACT AAT AAA AAT CAA TTT GGA ATA TAT AAT GAT AAA CTT AGT Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser 885 890 895	2688
GAA GTT AAT ATA TCT CAA AAT GAT TAC ATT ATA TAT GAT AAT AAA TAT Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr 900 905 910	2736
AAA AAT TTT AGT ATT AGT TTT TGG GTA AGA ATT CCT AAC TAT GAT AAT Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn 915 920 925	2784
AAG ATA GTA AAT GTT AAT AAT GAA TAC ACT ATA ATA AAT TGT ATG AGG Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg 930 935 940	2832
GAT AAT AAT TCA GGA TGG AAA GTA TCT CTT AAT CAT AAT GAA ATA ATT Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile 945 950 955 960	2880

TGG ACA TTG CAA GAT AAT TCA GGA ATT AAT CAA AAA TTA GCA TTT AAC Trp Thr Leu Gln Asp Asn Ser Gly Ile Asn Gln Lys Leu Ala Phe Asn 965 970 975	2928
TAT GGT AAC GCA AAT GGT ATT TCT GAT TAT ATA AAT AAG TGG ATT TTT Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe 980 985 990	2976
GTA ACT ATA ACT AAT GAT AGA TTA GGA GAT TCT AAA CTT TAT ATT AAT Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn 995 1000 1005	3024
GGA AAT TTA ATA GAT AAA AAA TCA ATT TTA AAT TTA GGT AAT ATT CAT Gly Asn Leu Ile Asp Lys Lys Ser Ile Leu Asn Leu Gly Asn Ile His 1010 1015 1020	3072
GTT AGT GAC AAT ATA TTA TTT AAA ATA GTT AAT TGT AGT TAT ACA AGA Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg 1025 1030 1035 1040	3120
TAT ATT GGT ATT AGA TAT TTT AAT ATT TTT GAT AAA GAA TTA GAT GAA Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu 1045 1050 1055	3168
ACA GAA ATT CAA ACT TTA TAT AAC AAT GAA CCT AAT GCA AAT ATT TTA Thr Glu Ile Gln Thr Leu Tyr Asn Asn Glu Pro Asn Ala Asn Ile Leu 1060 1065 1070	3216
AAG GAT TTT TGG GGA AAT TAT TTG CTT TAT GAC AAA GAA TAC TAT TTA Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu 1075 1080 1085	3264
TTA AAT GTG TTA AAA CCA AAT AAC TTT ATT AAT AGG AGA ACA GAT TCT Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asn Arg Arg Thr Asp Ser 1090 1095 1100	3312
ACT TTA AGC ATT AAT AAT ATA AGA AGC ACT ATT CTT TTA GCT AAT AGA Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg 1105 1110 1115 1120	3360
TTA TAT AGT GGA ATA AAA GTT AAA ATA CAA AGA GTT AAT AAT AGT AGT Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser 1125 1130 1135	3408
ACT AAC GAT AAT CTT GTT AGA AAG AAT GAT CAG GTA TAT ATT AAT TTT Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe 1140 1145 1150	3456
GTA GCC AGC AAA ACT CAC TTA CTT CCA TTA TAT GCT GAT ACA GCT ACC Val Ala Ser Lys Thr His Leu Leu Pro Leu Tyr Ala Asp Thr Ala Thr 1155 1160 1165	3504
ACA AAT AAA GAG AAA ACA ATA AAA ATA TCA TCA TCT GGC AAT AGA TTT Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe 1170 1175 1180	3552
AAT CAA GTA GTA GTT ATG AAT TCA GTA GGA TGT ACA ATG AAT TTT AAA Asn Gln Val Val Val Met Asn Ser Val Gly Cys Thr Met Asn Phe Lys 1185 1190 1195 1200	3600

AAT AAT AAT GGA AAT AAT ATT GGG TTG TTA GGT TTC AAG GCA GAT ACT	3648
Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala Asp Thr	
1205 1210 1215	
GTA GTT GCT AGT ACT TGG TAT TAT ACA CAT ATG AGA GAT AAT ACA AAC	3696
Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp Asn Thr Asn	
1220 1225 1230	
AGC AAT GGA TTT TTT TGG AAC TTT ATT TCT GAA GAA CAT GGA TGG CAA	3744
Ser Asn Gly Phe Phe Trp Asn Phe Ile Ser Glu Glu His Gly Trp Gln	
1235 1240 1245	
GAA AAA TAA	3753
Glu Lys	
1250	

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1250 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Met	Pro	Thr	Ile	Asn	Ser	Phe	Asn	Tyr	Asn	Asp	Pro	Val	Asn	Asn	Arg
1				5					10						15
Thr	Ile	Leu	Tyr	Ile	Lys	Pro	Gly	Gly	Cys	Gln	Gln	Phe	Tyr	Lys	Ser
		20						25					30		
Phe	Asn	Ile	Met	Lys	Asn	Ile	Trp	Ile	Ile	Pro	Glu	Arg	Asn	Val	Ile
		35					40					45			
Gly	Thr	Ile	Pro	Gln	Asp	Phe	Leu	Pro	Pro	Thr	Ser	Leu	Lys	Asn	Gly
	50					55					60				
Asp	Ser	Ser	Tyr	Tyr	Asp	Pro	Asn	Tyr	Leu	Gln	Ser	Asp	Gln	Glu	Lys
65					70					75					80
Asp	Lys	Phe	Leu	Lys	Ile	Val	Thr	Lys	Ile	Phe	Asn	Arg	Ile	Asn	Asp
				85					90					95	
Asn	Leu	Ser	Gly	Arg	Ile	Leu	Leu	Glu	Glu	Leu	Ser	Lys	Ala	Asn	Pro
			100					105					110		
Tyr	Leu	Gly	Asn	Asp	Asn	Thr	Pro	Asp	Gly	Asp	Phe	Ile	Ile	Asn	Asp
		115					120					125			
Ala	Ser	Ala	Val	Pro	Ile	Gln	Phe	Ser	Asn	Gly	Ser	Gln	Ser	Ile	Leu
		130				135						140			
Leu	Pro	Asn	Val	Ile	Ile	Met	Gly	Ala	Glu	Pro	Asp	Leu	Phe	Glu	Thr
145					150					155					160
Asn	Ser	Ser	Asn	Ile	Ser	Leu	Arg	Asn	Asn	Tyr	Met	Pro	Ser	Asn	His
				165					170					175	
Gly	Phe	Gly	Ser	Ile	Ala	Ile	Val	Thr	Phe	Ser	Pro	Glu	Tyr	Ser	Phe
			180					185					190		

Arg Phe Lys Asp Asn Ser Met Asn Glu Phe Ile Gln Asp Pro Ala Leu  
 195 200 205  
 Thr Leu Met His Glu Leu Ile His Ser Leu His Gly Leu Tyr Gly Ala  
 210 215 220  
 Lys Gly Ile Thr Thr Lys Tyr Thr Ile Thr Gln Lys Gln Asn Pro Leu  
 225 230 235 240  
 Ile Thr Asn Ile Arg Gly Thr Asn Ile Glu Glu Phe Leu Thr Phe Gly  
 245 250 255  
 Gly Thr Asp Leu Asn Ile Ile Thr Ser Ala Gln Ser Asn Asp Ile Tyr  
 260 265 270  
 Thr Asn Leu Leu Ala Asp Tyr Lys Lys Ile Ala Ser Lys Leu Ser Lys  
 275 280 285  
 Val Gln Val Ser Asn Pro Leu Leu Asn Pro Tyr Lys Asp Val Phe Glu  
 290 295 300  
 Ala Lys Tyr Gly Leu Asp Lys Asp Ala Ser Gly Ile Tyr Ser Val Asn  
 305 310 315 320  
 Ile Asn Lys Phe Asn Asp Ile Phe Lys Lys Leu Tyr Ser Phe Thr Glu  
 325 330 335  
 Phe Asp Leu Ala Thr Lys Phe Gln Val Lys Cys Arg Gln Thr Tyr Ile  
 340 345 350  
 Gly Gln Tyr Lys Tyr Phe Lys Leu Ser Asn Leu Leu Asn Asp Ser Ile  
 355 360 365  
 Tyr Asn Ile Ser Glu Gly Tyr Asn Ile Asn Asn Leu Lys Val Asn Phe  
 370 375 380  
 Arg Gly Gln Asn Ala Asn Leu Asn Pro Arg Ile Ile Thr Pro Ile Thr  
 385 390 395 400  
 Gly Arg Gly Leu Val Lys Lys Ile Ile Arg Phe Cys Lys Asn Ile Val  
 405 410 415  
 Ser Val Lys Gly Ile Arg Lys Ser Ile Cys Ile Glu Ile Asn Asn Gly  
 420 425 430  
 Glu Leu Phe Phe Val Ala Ser Glu Asn Ser Tyr Asn Asp Asp Asn Ile  
 435 440 445  
 Asn Thr Pro Lys Glu Ile Asp Asp Thr Val Thr Ser Asn Asn Asn Tyr  
 450 455 460  
 Glu Asn Asp Leu Asp Gln Val Ile Leu Asn Phe Asn Ser Glu Ser Ala  
 465 470 475 480  
 Pro Gly Leu Ser Asp Glu Lys Leu Asn Leu Thr Ile Gln Asn Asp Ala  
 485 490 495  
 Tyr Ile Pro Lys Tyr Asp Ser Asn Gly Thr Ser Asp Ile Glu Gln His  
 500 505 510  
 Asp Val Asn Glu Leu Asn Val Phe Phe Tyr Leu Asp Ala Gln Lys Val  
 515 520 525

Pro Glu Gly Glu Asn Asn Val Asn Leu Thr Ser Ser Ile Asp Thr Ala  
 530 535 540  
 Leu Leu Glu Gln Pro Lys Ile Tyr Thr Phe Phe Ser Ser Glu Phe Ile  
 545 550 555 560  
 Asn Asn Val Asn Lys Pro Val Gln Ala Ala Leu Phe Val Ser Trp Ile  
 565 570 575  
 Gln Gln Val Leu Val Asp Phe Thr Thr Glu Ala Asn Gln Lys Ser Thr  
 580 585 590  
 Val Asp Lys Ile Ala Asp Ile Ser Ile Val Val Pro Tyr Ile Gly Leu  
 595 600 605  
 Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Phe Lys Asp Ala  
 610 615 620  
 Leu Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Glu Pro Glu Leu  
 625 630 635 640  
 Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser  
 645 650 655  
 Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Asn Ala Leu Lys  
 660 665 670  
 Glu Arg Asp Glu Lys Trp Lys Glu Val Tyr Ser Phe Ile Val Ser Asn  
 675 680 685  
 Trp Met Thr Lys Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln Met  
 690 695 700  
 Tyr Gln Ala Leu Gln Asn Gln Val Asn Ala Leu Lys Ala Ile Ile Glu  
 705 710 715 720  
 Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Glu Lys Asn Glu Leu Thr Asn  
 725 730 735  
 Lys Tyr Asp Ile Glu Gln Ile Glu Asn Glu Leu Asn Gln Lys Val Ser  
 740 745 750  
 Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser  
 755 760 765  
 Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu  
 770 775 780  
 Tyr Asp Glu Asn Val Lys Thr Tyr Leu Leu Asp Tyr Ile Ile Lys His  
 785 790 795 800  
 Gly Ser Ile Leu Gly Glu Ser Gln Gln Glu Leu Asn Ser Met Val Ile  
 805 810 815  
 Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp  
 820 825 830  
 Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys  
 835 840 845  
 Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp  
 850 855 860

Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys  
 865 870 875 880  
 Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser  
 885 890 895  
 Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr  
 900 905 910  
 Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn  
 915 920 925  
 Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg  
 930 935 940  
 Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile  
 945 950 955 960  
 Trp Thr Leu Gln Asp Asn Ser Gly Ile Asn Gln Lys Leu Ala Phe Asn  
 965 970 975  
 Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe  
 980 985 990  
 Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn  
 995 1000 1005  
 Gly Asn Leu Ile Asp Lys Lys Ser Ile Leu Asn Leu Gly Asn Ile His  
 1010 1015 1020  
 Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg  
 1025 1030 1035 1040  
 Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu  
 1045 1050 1055  
 Thr Glu Ile Gln Thr Leu Tyr Asn Asn Glu Pro Asn Ala Asn Ile Leu  
 1060 1065 1070  
 Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu  
 1075 1080 1085  
 Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asn Arg Arg Thr Asp Ser  
 1090 1095 1100  
 Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg  
 1105 1110 1115 1120  
 Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser  
 1125 1130 1135  
 Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe  
 1140 1145 1150  
 Val Ala Ser Lys Thr His Leu Leu Pro Leu Tyr Ala Asp Thr Ala Thr  
 1155 1160 1165  
 Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe  
 1170 1175 1180  
 Asn Gln Val Val Val Met Asn Ser Val Gly Cys Thr Met Asn Phe Lys  
 1185 1190 1195 1200



Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala Asp Thr  
1205 1210 1215

Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp Asn Thr Asn  
1220 1225 1230

Ser Asn Gly Phe Phe Trp Asn Phe Ile Ser Glu Glu His Gly Trp Gln  
1235 1240 1245

Glu Lys  
1250

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 3759 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 1..3756

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

ATG CCA AAA ATT AAT AGT TTT AAT TAT AAT GAT CCT GTT AAT GAT AGA	48
Met Pro Lys Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp Arg	
1 5 10 15	
ACA ATT TTA TAT ATT AAA CCA GGC GGT TGT CAA GAA TTT TAT AAA TCA	96
Thr Ile Leu Tyr Ile Lys Pro Gly Gly Cys Gln Glu Phe Tyr Lys Ser	
20 25 30	
TTT AAT ATT ATG AAA AAT ATT TGG ATA ATT CCA GAG AGA AAT GTA ATT	144
Phe Asn Ile Met Lys Asn Ile Trp Ile Ile Pro Glu Arg Asn Val Ile	
35 40 45	
GGT ACA ACC CCC CAA GAT TTT CAT CCG CCT ACT TCA TTA AAA AAT GGA	192
Gly Thr Thr Pro Gln Asp Phe His Pro Pro Thr Ser Leu Lys Asn Gly	
50 55 60	
GAT AGT AGT TAT TAT GAC CCT AAT TAT TTA CAA AGT GAT GAA GAA AAG	240
Asp Ser Ser Tyr Tyr Asp Pro Asn Tyr Leu Gln Ser Asp Glu Glu Lys	
65 70 75 80	
GAT AGA TTT TTA AAA ATA GTC ACA AAA ATA TTT AAT AGA ATA AAT AAT	288
Asp Arg Phe Leu Lys Ile Val Thr Lys Ile Phe Asn Arg Ile Asn Asn	
85 90 95	
AAT CTT TCA GGA GGG ATT TTA TTA GAA GAA CTG TCA AAA GCT AAT CCA	336
Asn Leu Ser Gly Gly Ile Leu Leu Glu Glu Leu Ser Lys Ala Asn Pro	
100 105 110	
TAT TTA GGG AAT GAT AAT ACT CCA GAT AAT CAA TTC CAT ATT GGT GAT	384
Tyr Leu Gly Asn Asp Asn Thr Pro Asp Asn Gln Phe His Ile Gly Asp	
115 120 125	

GCA TCA GCA GTT GAG ATT AAA TTC TCA AAT GGT AGC CAA GAC ATA CTA Ala Ser Ala Val Glu Ile Lys Phe Ser Asn Gly Ser Gln Asp Ile Leu 130 135 140	432
TTA CCT AAT GTT ATT ATA ATG GGA GCA GAG CCT GAT TTA TTT GAA ACT Leu Pro Asn Val Ile Ile Met Gly Ala Glu Pro Asp Leu Phe Glu Thr 145 150 155 160	480
AAC AGT TCC AAT ATT TCT CTA AGA AAT AAT TAT ATG CCA AGC AAT CAC Asn Ser Ser Asn Ile Ser Leu Arg Asn Asn Tyr Met Pro Ser Asn His 165 170 175	528
GGT TTT GGA TCA ATA GCT ATA GTA ACA TTC TCA CCT GAA TAT TCT TTT Gly Phe Gly Ser Ile Ala Ile Val Thr Phe Ser Pro Glu Tyr Ser Phe 180 185 190	576
AGA TTT AAT GAT AAT AGT ATG AAT GAA TTT ATT CAA GAT CCT GCT CTT Arg Phe Asn Asp Asn Ser Met Asn Glu Phe Ile Gln Asp Pro Ala Leu 195 200 205	624
ACA TTA ATG CAT GAA TTA ATA CAT TCA TTA CAT GGA CTA TAT GGG GCT Thr Leu Met His Glu Leu Ile His Ser Leu His Gly Leu Tyr Gly Ala 210 215 220	672
AAA GGG ATT ACT ACA AAG TAT ACT ATA ACA CAA AAA CAA AAT CCC CTA Lys Gly Ile Thr Thr Lys Tyr Thr Ile Thr Gln Lys Gln Asn Pro Leu 225 230 235 240	720
ATA ACA AAT ATA AGA GGT ACA AAT ATT GAA GAA TTC TTA ACT TTT GGA Ile Thr Asn Ile Arg Gly Thr Asn Ile Glu Glu Phe Leu Thr Phe Gly 245 250 255	768
GGT ACT GAT TTA AAC ATT ATT ACT AGT GCT CAG TCC AAT GAT ATC TAT Gly Thr Asp Leu Asn Ile Ile Thr Ser Ala Gln Ser Asn Asp Ile Tyr 260 265 270	816
ACT AAT CTT CTA GCT GAT TAT AAA AAA ATA GCG TCT AAA CTT AGC AAA Thr Asn Leu Leu Ala Asp Tyr Lys Lys Ile Ala Ser Lys Leu Ser Lys 275 280 285	864
GTA CAA GTA TCT AAT CCA CTA CTT AAT CCT TAT AAA GAT GTT TTT GAA Val Gln Val Ser Asn Pro Leu Leu Asn Pro Tyr Lys Asp Val Phe Glu 290 295 300	912
GCA AAG TAT GGA TTA GAT AAA GAT GCT AGC GGA ATT TAT TCG GTA AAT Ala Lys Tyr Gly Leu Asp Lys Asp Ala Ser Gly Ile Tyr Ser Val Asn 305 310 315 320	960
ATA AAC AAA TTT AAT GAT ATT TTT AAA AAA TTA TAC AGC TTT ACG GAA Ile Asn Lys Phe Asn Asp Ile Phe Lys Lys Leu Tyr Ser Phe Thr Glu 325 330 335	1008
TTT GAT TTA GCA ACT AAA TTT CAA GTT AAA TGT AGG CAA ACT TAT ATT Phe Asp Leu Ala Thr Lys Phe Gln Val Lys Cys Arg Gln Thr Tyr Ile 340 345 350	1056
GGA CAG TAT AAA TAC TTC AAA CTT TCA AAC TTG TTA AAT GAT TCT ATT Gly Gln Tyr Lys Tyr Phe Lys Leu Ser Asn Leu Leu Asn Asp Ser Ile 355 360 365	1104

TAT AAT ATA TCA GAA GGC TAT AAT ATA AAT AAT TTA AAG GTA AAT TTT Tyr Asn Ile Ser Glu Gly Tyr Asn Ile Asn Asn Leu Lys Val Asn Phe 370 375 380	1152
AGA GGA CAG AAT GCA AAT TTA AAT CCT AGA ATT ATT ACA CCA ATT ACA Arg Gly Gln Asn Ala Asn Leu Asn Pro Arg Ile Ile Thr Pro Ile Thr 385 390 395 400	1200
GGT AGA GGA CTA GTA AAA AAA ATC ATT AGA TTT TGT AAA AAT ATT GTT Gly Arg Gly Leu Val Lys Lys Ile Ile Arg Phe Cys Lys Asn Ile Val 405 410 415	1248
TCT GTA AAA GGC ATA AGG AAA TCA ATA TGT ATC GAA ATA AAT AAT GGT Ser Val Lys Gly Ile Arg Lys Ser Ile Cys Ile Glu Ile Asn Asn Gly 420 425 430	1296
GAG TTA TTT TTT GTG GCT TCC GAG AAT AGT TAT AAT GAT GAT AAT ATA Glu Leu Phe Phe Val Ala Ser Glu Asn Ser Tyr Asn Asp Asp Asn Ile 435 440 445	1344
AAT ACT CCT AAA GAA ATT GAC GAT ACA GTA ACT TCA AAT AAT AAT TAT Asn Thr Pro Lys Glu Ile Asp Asp Thr Val Thr Ser Asn Asn Asn Tyr 450 455 460	1392
GAA AAT GAT TTA GAT CAG GTT ATT TTA AAT TTT AAT AGT GAA TCA GCA Glu Asn Asp Leu Asp Gln Val Ile Leu Asn Phe Asn Ser Glu Ser Ala 465 470 475 480	1440
CCT GGA CTT TCA GAT GAA AAA TTA AAT TTA ACT ATC CAA AAT GAT GCT Pro Gly Leu Ser Asp Glu Lys Leu Asn Leu Thr Ile Gln Asn Asp Ala 485 490 495	1488
TAT ATA CCA AAA TAT GAT TCT AAT GGA ACA AGT GAT ATA GAA CAA CAT Tyr Ile Pro Lys Tyr Asp Ser Asn Gly Thr Ser Asp Ile Glu Gln His 500 505 510	1536
GAT GTT AAT GAA CTT AAT GTA TTT TTC TAT TTA GAT GCA CAG AAA GTG Asp Val Asn Glu Leu Asn Val Phe Phe Tyr Leu Asp Ala Gln Lys Val 515 520 525	1584
CCC GAA GGT GAA AAT AAT GTC AAT CTC ACC TCT TCA ATT GAT ACA GCA Pro Glu Gly Glu Asn Asn Val Asn Leu Thr Ser Ser Ile Asp Thr Ala 530 535 540	1632
TTA TTA GAA CAA CCT AAA ATA TAT ACA TTT TTT TCA TCA GAA TTT ATT Leu Leu Glu Gln Pro Lys Ile Tyr Thr Phe Phe Ser Ser Glu Phe Ile 545 550 555 560	1680
AAT AAT GTC AAT AAA CCT GTG CAA GCA GCA TTA TTT GTA AGC TGG ATA Asn Asn Val Asn Lys Pro Val Gln Ala Ala Leu Phe Val Ser Trp Ile 565 570 575	1728
CAA CAA GTG TTA GTA GAT TTT ACT ACT GAA GCT AAC CAA AAA AGT ACT Gln Gln Val Leu Val Asp Phe Thr Thr Glu Ala Asn Gln Lys Ser Thr 580 585 590	1776
GTT GAT AAA ATT GCA GAT ATT TCT ATA GTT GTT CCA TAT ATA GGT CTT Val Asp Lys Ile Ala Asp Ile Ser Ile Val Val Pro Tyr Ile Gly Leu 595 600 605	1824

GCT TTA AAT ATA GGA AAT GAA GCA CAA AAA GGA AAT TTT AAA GAT GCA Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Phe Lys Asp Ala 610 615 620	1872
CTT GAA TTA TTA GGA GCA GGT ATT TTA TTA GAA TTT GAA CCC GAG CTT Leu Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Glu Pro Glu Leu 625 630 635 640	1920
TTA ATT CCT ACA ATT TTA GTA TTC ACG ATA AAA TCT TTT TTA GGT TCA Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser 645 650 655	1968
TCT GAT AAT AAA AAT AAA GTT ATT AAA GCA ATA AAT AAT GCA TTG AAA Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Asn Ala Leu Lys 660 665 670	2016
GAA AGA GAT GAA AAA TGG AAA GAA GTA TAT AGT TTT ATA GTA TCG AAT Glu Arg Asp Glu Lys Trp Lys Glu Val Tyr Ser Phe Ile Val Ser Asn 675 680 685	2064
TGG ATG ACT AAA ATT AAT ACA CAA TTT AAT AAA AGA AAA GAA CAA ATG Trp Met Thr Lys Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln Met 690 695 700	2112
TAT CAA GCT TTA CAA AAT CAA GTA AAT GCA ATT AAA ACA ATA ATA GAA Tyr Gln Ala Leu Gln Asn Gln Val Asn Ala Ile Lys Thr Ile Ile Glu 705 710 715 720	2160
TCT AAG TAT AAT AGT TAT ACT TTA GAG GAA AAA AAT GAG CTT ACA AAT Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Glu Lys Asn Glu Leu Thr Asn 725 730 735	2208
AAA TAT GAT ATT AAG CAA ATA GAA AAT GAA CTT AAT CAA AAG GTT TCT Lys Tyr Asp Ile Lys Gln Ile Glu Asn Glu Leu Asn Gln Lys Val Ser 740 745 750	2256
ATA GCA ATG AAT AAT ATA GAC AGG TTC TTA ACT GAA AGT TCT ATA TCC Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser 755 760 765	2304
TAT TTA ATG AAA TTA ATA AAT GAA GTA AAA ATT AAT AAA TTA AGA GAA Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu 770 775 780	2352
TAT GAT GAG AAT GTC AAA ACG TAT TTA TTG AAT TAT ATT ATA CAA CAT Tyr Asp Glu Asn Val Lys Thr Tyr Leu Leu Asn Tyr Ile Ile Gln His 785 790 795 800	2400
GGA TCA ATC TTG GGA GAG AGT CAG CAA GAA CTA AAT TCT ATG GTA ACT Gly Ser Ile Leu Gly Glu Ser Gln Gln Glu Leu Asn Ser Met Val Thr 805 810 815	2448
GAT ACC CTA AAT AAT AGT ATT CCT TTT AAG CTT TCT TCT TAT ACA GAT Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp 820 825 830	2496
GAT AAA ATT TTA ATT TCA TAT TTT AAT AAA TTC TTT AAG AGA ATT AAA Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys 835 840 845	2544

AGT AGT TCA GTT TTA AAT ATG AGA TAT AAA AAT GAT AAA TAC GTA GAT Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp 850 855 860	2592
ACT TCA GGA TAT GAT TCA AAT ATA AAT ATT AAT GGA GAT GTA TAT AAA Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys 865 870 875 880	2640
TAT CCA ACT AAT AAA AAT CAA TTT GGA ATA TAT AAT GAT AAA CTT AGT Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser 885 890 895	2688
GAA GTT AAT ATA TCT CAA AAT GAT TAC ATT ATA TAT GAT AAT AAA TAT Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr 900 905 910	2736
AAA AAT TTT AGT ATT AGT TTT TGG GTA AGA ATT CCT AAC TAT GAT AAT Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn 915 920 925	2784
AAG ATA GTA AAT GTT AAT AAT GAA TAC ACT ATA ATA AAT TGT ATG AGA Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg 930 935 940	2832
GAT AAT AAT TCA GGA TGG AAA GTA TCT CTT AAT CAT AAT GAA ATA ATT Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile 945 950 955 960	2880
TGG ACA TTG CAA GAT AAT GCA GGA ATT AAT CAA AAA TTA GCA TTT AAC Trp Thr Leu Gln Asp Asn Ala Gly Ile Asn Gln Lys Leu Ala Phe Asn 965 970 975	2928
TAT GGT AAC GCA AAT GGT ATT TCT GAT TAT ATA AAT AAG TGG ATT TTT Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe 980 985 990	2976
GTA ACT ATA ACT AAT GAT AGA TTA GGA GAT TCT AAA CTT TAT ATT AAT Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn 995 1000 1005	3024
GGA AAT TTA ATA GAT CAA AAA TCA ATT TTA AAT TTA GGT AAT ATT CAT Gly Asn Leu Ile Asp Gln Lys Ser Ile Leu Asn Leu Gly Asn Ile His 1010 1015 1020	3072
GTT AGT GAC AAT ATA TTA TTT AAA ATA GTT AAT TGT AGT TAT ACA AGA Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg 1025 1030 1035 1040	3120
TAT ATT GGT ATT AGA TAT TTT AAT ATT TTT GAT AAA GAA TTA GAT GAA Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu 1045 1050 1055	3168
ACA GAA ATT CAA ACT TTA TAT AGC AAT GAA CCT AAT ACA AAT ATT TTG Thr Glu Ile Gln Thr Leu Tyr Ser Asn Glu Pro Asn Thr Asn Ile Leu 1060 1065 1070	3216
AAG GAT TTT TGG GGA AAT TAT TTG CTT TAT GAC AAA GAA TAC TAT TTA Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu 1075 1080 1085	3264

TTA AAT GTG TTA AAA CCA AAT AAC TTT ATT GAT AGG AGA AAA GAT TCT Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asp Arg Arg Lys Asp Ser 1090 1095 1100	3312
ACT TTA AGC ATT AAT AAT ATA AGA AGC ACT ATT CTT TTA GCT AAT AGA Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg 1105 1110 1115 1120	3360
TTA TAT AGT GGA ATA AAA GTT AAA ATA CAA AGA GTT AAT AAT AGT AGT Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser 1125 1130 1135	3408
ACT AAC GAT AAT CTT GTT AGA AAG AAT GAT CAG GTA TAT ATT AAT TTT Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe 1140 1145 1150	3456
GTA GCC AGC AAA ACT CAC TTA TTT CCA TTA TAT GCT GAT ACA GCT ACC Val Ala Ser Lys Thr His Leu Phe Pro Leu Tyr Ala Asp Thr Ala Thr 1155 1160 1165	3504
ACA AAT AAA GAG AAA ACA ATA AAA ATA TCA TCA TCT GGC AAT AGA TTT Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe 1170 1175 1180	3552
AAT CAA GTA GTA GTT ATG AAT TCA GTA GGA AAT AAT TGT ACA ATG AAT Asn Gln Val Val Val Met Asn Ser Val Gly Asn Asn Cys Thr Met Asn 1185 1190 1195 1200	3600
TTT AAA AAT AAT AAT GGA AAT AAT ATT GGG TTG TTA GGT TTC AAG GCA Phe Lys Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala 1205 1210 1215	3648
GAT ACT GTA GTT GCT AGT ACT TGG TAT TAT ACA CAT ATG AGA GAT CAT Asp Thr Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp His 1220 1225 1230	3696
ACA AAC AGC AAT GGA TGT TTT TGG AAC TTT ATT TCT GAA GAA CAT GGA Thr Asn Ser Asn Gly Cys Phe Trp Asn Phe Ile Ser Glu Glu His Gly 1235 1240 1245	3744
TGG CAA GAA AAA TAA Trp Gln Glu Lys 1250	3759

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1252 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Met Pro Lys Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp Arg 1 5 10 15
Thr Ile Leu Tyr Ile Lys Pro Gly Gly Cys Gln Glu Phe Tyr Lys Ser 20 25 30

Phe Asn Ile Met Lys Asn Ile Trp Ile Ile Pro Glu Arg Asn Val Ile  
 35 40 45  
 Gly Thr Thr Pro Gln Asp Phe His Pro Pro Thr Ser Leu Lys Asn Gly  
 50 55 60  
 Asp Ser Ser Tyr Tyr Asp Pro Asn Tyr Leu Gln Ser Asp Glu Glu Lys  
 65 70 75 80  
 Asp Arg Phe Leu Lys Ile Val Thr Lys Ile Phe Asn Arg Ile Asn Asn  
 85 90 95  
 Asn Leu Ser Gly Gly Ile Leu Leu Glu Glu Leu Ser Lys Ala Asn Pro  
 100 105 110  
 Tyr Leu Gly Asn Asp Asn Thr Pro Asp Asn Gln Phe His Ile Gly Asp  
 115 120 125  
 Ala Ser Ala Val Glu Ile Lys Phe Ser Asn Gly Ser Gln Asp Ile Leu  
 130 135 140  
 Leu Pro Asn Val Ile Ile Met Gly Ala Glu Pro Asp Leu Phe Glu Thr  
 145 150 155 160  
 Asn Ser Ser Asn Ile Ser Leu Arg Asn Asn Tyr Met Pro Ser Asn His  
 165 170 175  
 Gly Phe Gly Ser Ile Ala Ile Val Thr Phe Ser Pro Glu Tyr Ser Phe  
 180 185 190  
 Arg Phe Asn Asp Asn Ser Met Asn Glu Phe Ile Gln Asp Pro Ala Leu  
 195 200 205  
 Thr Leu Met His Glu Leu Ile His Ser Leu His Gly Leu Tyr Gly Ala  
 210 215 220  
 Lys Gly Ile Thr Thr Lys Tyr Thr Ile Thr Gln Lys Gln Asn Pro Leu  
 225 230 235 240  
 Ile Thr Asn Ile Arg Gly Thr Asn Ile Glu Glu Phe Leu Thr Phe Gly  
 245 250 255  
 Gly Thr Asp Leu Asn Ile Ile Thr Ser Ala Gln Ser Asn Asp Ile Tyr  
 260 265 270  
 Thr Asn Leu Leu Ala Asp Tyr Lys Lys Ile Ala Ser Lys Leu Ser Lys  
 275 280 285  
 Val Gln Val Ser Asn Pro Leu Leu Asn Pro Tyr Lys Asp Val Phe Glu  
 290 295 300  
 Ala Lys Tyr Gly Leu Asp Lys Asp Ala Ser Gly Ile Tyr Ser Val Asn  
 305 310 315 320  
 Ile Asn Lys Phe Asn Asp Ile Phe Lys Lys Leu Tyr Ser Phe Thr Glu  
 325 330 335  
 Phe Asp Leu Ala Thr Lys Phe Gln Val Lys Cys Arg Gln Thr Tyr Ile  
 340 345 350  
 Gly Gln Tyr Lys Tyr Phe Lys Leu Ser Asn Leu Leu Asn Asp Ser Ile  
 355 360 365

Tyr Asn Ile Ser Glu Gly Tyr Asn Ile Asn Asn Leu Lys Val Asn Phe  
 370 375 380  
 Arg Gly Gln Asn Ala Asn Leu Asn Pro Arg Ile Ile Thr Pro Ile Thr  
 385 390 395 400  
 Gly Arg Gly Leu Val Lys Lys Ile Ile Arg Phe Cys Lys Asn Ile Val  
 405 410 415  
 Ser Val Lys Gly Ile Arg Lys Ser Ile Cys Ile Glu Ile Asn Asn Gly  
 420 425 430  
 Glu Leu Phe Phe Val Ala Ser Glu Asn Ser Tyr Asn Asp Asp Asn Ile  
 435 440 445  
 Asn Thr Pro Lys Glu Ile Asp Asp Thr Val Thr Ser Asn Asn Asn Tyr  
 450 455 460  
 Glu Asn Asp Leu Asp Gln Val Ile Leu Asn Phe Asn Ser Glu Ser Ala  
 465 470 475 480  
 Pro Gly Leu Ser Asp Glu Lys Leu Asn Leu Thr Ile Gln Asn Asp Ala  
 485 490 495  
 Tyr Ile Pro Lys Tyr Asp Ser Asn Gly Thr Ser Asp Ile Glu Gln His  
 500 505 510  
 Asp Val Asn Glu Leu Asn Val Phe Phe Tyr Leu Asp Ala Gln Lys Val  
 515 520 525  
 Pro Glu Gly Glu Asn Asn Val Asn Leu Thr Ser Ser Ile Asp Thr Ala  
 530 535 540  
 Leu Leu Glu Gln Pro Lys Ile Tyr Thr Phe Phe Ser Ser Glu Phe Ile  
 545 550 555 560  
 Asn Asn Val Asn Lys Pro Val Gln Ala Ala Leu Phe Val Ser Trp Ile  
 565 570 575  
 Gln Gln Val Leu Val Asp Phe Thr Thr Glu Ala Asn Gln Lys Ser Thr  
 580 585 590  
 Val Asp Lys Ile Ala Asp Ile Ser Ile Val Val Pro Tyr Ile Gly Leu  
 595 600 605  
 Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Phe Lys Asp Ala  
 610 615 620  
 Leu Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Glu Pro Glu Leu  
 625 630 635 640  
 Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser  
 645 650 655  
 Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Asn Ala Leu Lys  
 660 665 670  
 Glu Arg Asp Glu Lys Trp Lys Glu Val Tyr Ser Phe Ile Val Ser Asn  
 675 680 685  
 Trp Met Thr Lys Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln Met  
 690 695 700



Tyr Gln Ala Leu Gln Asn Gln Val Asn Ala Ile Lys Thr Ile Ile Glu  
 705 710 715 720  
 Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Glu Lys Asn Glu Leu Thr Asn  
 725 730 735  
 Lys Tyr Asp Ile Lys Gln Ile Glu Asn Glu Leu Asn Gln Lys Val Ser  
 740 745 750  
 Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser  
 755 760 765  
 Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu  
 770 775 780  
 Tyr Asp Glu Asn Val Lys Thr Tyr Leu Leu Asn Tyr Ile Ile Gln His  
 785 790 795 800  
 Gly Ser Ile Leu Gly Glu Ser Gln Gln Glu Leu Asn Ser Met Val Thr  
 805 810 815  
 Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp  
 820 825 830  
 Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys  
 835 840 845  
 Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp  
 850 855 860  
 Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys  
 865 870 875 880  
 Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser  
 885 890 895  
 Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr  
 900 905 910  
 Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn  
 915 920 925  
 Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg  
 930 935 940  
 Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile  
 945 950 955 960  
 Trp Thr Leu Gln Asp Asn Ala Gly Ile Asn Gln Lys Leu Ala Phe Asn  
 965 970 975  
 Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe  
 980 985 990  
 Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn  
 995 1000 1005  
 Gly Asn Leu Ile Asp Gln Lys Ser Ile Leu Asn Leu Gly Asn Ile His  
 1010 1015 1020  
 Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg  
 1025 1030 1035 1040

Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu  
 1045 1050 1055  
 Thr Glu Ile Gln Thr Leu Tyr Ser Asn Glu Pro Asn Thr Asn Ile Leu  
 1060 1065 1070  
 Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu  
 1075 1080 1085  
 Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asp Arg Arg Lys Asp Ser  
 1090 1095 1100  
 Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg  
 1105 1110 1115 1120  
 Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser  
 1125 1130 1135  
 Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe  
 1140 1145 1150  
 Val Ala Ser Lys Thr His Leu Phe Pro Leu Tyr Ala Asp Thr Ala Thr  
 1155 1160 1165  
 Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe  
 1170 1175 1180  
 Asn Gln Val Val Val Met Asn Ser Val Gly Asn Asn Cys Thr Met Asn  
 1185 1190 1195 1200  
 Phe Lys Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala  
 1205 1210 1215  
 Asp Thr Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp His  
 1220 1225 1230  
 Thr Asn Ser Asn Gly Cys Phe Trp Asn Phe Ile Ser Glu Glu His Gly  
 1235 1240 1245  
 Trp Gln Glu Lys  
 1250

(2) INFORMATION FOR SEQ ID NO:53:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1463 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "DNA"
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 108..1460

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATAACC ATG GGC CAT	116
Met Gly His	
1	
CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT	164
His His His His His His His His His Ser Ser Gly His Ile Glu Gly	
5 10 15	
CGT CAT ATG GCT AGC ATG GCT CTT TCT TCT TAT ACA GAT GAT AAA ATT	212
Arg His Met Ala Ser Met Ala Leu Ser Ser Tyr Thr Asp Asp Lys Ile	
20 25 30 35	
TTA ATT TCA TAT TTT AAT AAG TTC TTT AAG AGA ATT AAA AGT AGT TCT	260
Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys Ser Ser Ser	
40 45 50	
GTT TTA AAT ATG AGA TAT AAA AAT GAT AAA TAC GTA GAT ACT TCA GGA	308
Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp Thr Ser Gly	
55 60 65	
TAT GAT TCA AAT ATA AAT ATT AAT GGA GAT GTA TAT AAA TAT CCA ACT	356
Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys Tyr Pro Thr	
70 75 80	
AAT AAA AAT CAA TTT GGA ATA TAT AAT GAT AAA CTT AGT GAA GTT AAT	404
Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser Glu Val Asn	
85 90 95	
ATA TCT CAA AAT GAT TAC ATT ATA TAT GAT AAT AAA TAT AAA AAT TTT	452
Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr Lys Asn Phe	
100 105 110 115	
AGT ATT AGT TTT TGG GTA AGA ATT CCT AAC TAT GAT AAT AAG ATA GTA	500
Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn Lys Ile Val	
120 125 130	
AAT GTT AAT AAT GAA TAC ACT ATA ATA AAT TGT ATG AGG GAT AAT AAT	548
Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg Asp Asn Asn	
135 140 145	
TCA GGA TGG AAA GTA TCT CTT AAT CAT AAT GAA ATA ATT TGG ACA TTG	596
Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile Trp Thr Leu	
150 155 160	
CAA GAT AAT TCA GGA ATT AAT CAA AAA TTA GCA TTT AAC TAT GGT AAC	644
Gln Asp Asn Ser Gly Ile Asn Gln Lys Leu Ala Phe Asn Tyr Gly Asn	
165 170 175	
GCA AAT GGT ATT TCT GAT TAT ATA AAT AAG TGG ATT TTT GTA ACT ATA	692
Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile	
180 185 190 195	
ACT AAT GAT AGA TTA GGA GAT TCT AAA CTT TAT ATT AAT GGA AAT TTA	740
Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn Gly Asn Leu	
200 205 210	

ATA GAT AAA AAA TCA ATT TTA AAT TTA GGT AAT ATT CAT GTT AGT GAC Ile Asp Lys Lys Ser Ile Leu Asn Leu Gly Asn Ile His Val Ser Asp 215 220 225	788
AAT ATA TTA TTT AAA ATA GTT AAT TGT AGT TAT ACA AGA TAT ATT GGT Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg Tyr Ile Gly 230 235 240	836
ATT AGA TAT TTT AAT ATT TTT GAT AAA GAA TTA GAT GAA ACA GAA ATT Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu Thr Glu Ile 245 250 255	884
CAA ACT TTA TAT AAC AAT GAA CCT AAT GCA AAT ATT TTA AAG GAT TTT Gln Thr Leu Tyr Asn Asn Glu Pro Asn Ala Asn Ile Leu Lys Asp Phe 260 265 270 275	932
TGG GGA AAT TAT TTG CTT TAT GAC AAA GAA TAC TAT TTA TTA AAT GTG Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu Leu Asn Val 280 285 290	980
TTA AAA CCA AAT AAC TTT ATT AAT AGG AGA ACA GAT TCT ACT TTA AGC Leu Lys Pro Asn Asn Phe Ile Asn Arg Arg Thr Asp Ser Thr Leu Ser 295 300 305	1028
ATT AAT AAT ATA AGA AGC ACT ATT CTT TTA GCT AAT AGA TTA TAT AGT Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg Leu Tyr Ser 310 315 320	1076
GGA ATA AAA GTT AAA ATA CAA AGA GTT AAT AAT AGT AGT ACT AAC GAT Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser Thr Asn Asp 325 330 335	1124
AAT CTT GTT AGA AAG AAT GAT CAG GTA TAT ATT AAT TTT GTA GCC AGC Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe Val Ala Ser 340 345 350 355	1172
AAA ACT CAC TTA CTT CCA TTA TAT GCT GAT ACA GCT ACC ACA AAT AAA Lys Thr His Leu Leu Pro Leu Tyr Ala Asp Thr Ala Thr Thr Asn Lys 360 365 370	1220
GAG AAA ACA ATA AAA ATA TCA TCA TCT GGC AAT AGA TTT AAT CAA GTA Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe Asn Gln Val 375 380 385	1268
GTA GTT ATG AAT TCA GTA GGA AAT TGT ACA ATG AAT TTT AAA AAT AAT Val Val Met Asn Ser Val Gly Asn Cys Thr Met Asn Phe Lys Asn Asn 390 395 400	1316
AAT GGA AAT AAT ATT GGG TTG TTA GGT TTC AAG GCA GAT ACT GTA GTT Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala Asp Thr Val Val 405 410 415	1364
GCT AGT ACT TGG TAT TAT ACA CAT ATG AGA GAT AAT ACA AAC AGC AAT Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp Asn Thr Asn Ser Asn 420 425 430 435	1412
GGA TTT TTT TGG AAC TTT ATT TCT GAA GAA CAT GGA TGG CAA GAA AAA Gly Phe Phe Trp Asn Phe Ile Ser Glu Glu His Gly Trp Gln Glu Lys 440 445 450	1460
TAA	1463

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 451 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Met Gly His His His His His His His His His His Ser Ser Gly His  
1 5 10 15  
Ile Glu Gly Arg His Met Ala Ser Met Ala Leu Ser Ser Tyr Thr Asp  
20 25 30  
Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys  
35 40 45  
Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp  
50 55 60  
Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys  
65 70 75 80  
Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser  
85 90 95  
Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr  
100 105 110  
Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn  
115 120 125  
Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg  
130 135 140  
Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile  
145 150 155 160  
Trp Thr Leu Gln Asp Asn Ser Gly Ile Asn Gln Lys Leu Ala Phe Asn  
165 170 175  
Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe  
180 185 190  
Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn  
195 200 205  
Gly Asn Leu Ile Asp Lys Lys Ser Ile Leu Asn Leu Gly Asn Ile His  
210 215 220  
Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg  
225 230 235 240  
Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu  
245 250 255  
Thr Glu Ile Gln Thr Leu Tyr Asn Asn Glu Pro Asn Ala Asn Ile Leu  
260 265 270

Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu  
 275 280 285  
 Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asn Arg Arg Thr Asp Ser  
 290 295 300  
 Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg  
 305 310 315 320  
 Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser  
 325 330 335  
 Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe  
 340 345 350  
 Val Ala Ser Lys Thr His Leu Leu Pro Leu Tyr Ala Asp Thr Ala Thr  
 355 360 365  
 Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe  
 370 375 380  
 Asn Gln Val Val Val Met Asn Ser Val Gly Asn Cys Thr Met Asn Phe  
 385 390 395 400  
 Lys Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala Asp  
 405 410 415  
 Thr Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp Asn Thr  
 420 425 430  
 Asn Ser Asn Gly Phe Phe Trp Asn Phe Ile Ser Glu Glu His Gly Trp  
 435 440 445  
 Gln Glu Lys  
 450

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1472 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "DNA"

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 108..1463

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATAACC ATG GGC CAT	116
Met Gly His	
1	
CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT	164
His His His His His His His His Ser Ser Gly His Ile Glu Gly	
5 10 15	

CGT CAT ATG GCT AGC ATG GCT CTT TCT TCT TAT ACA GAT GAT AAA ATT	212
Arg His Met Ala Ser Met Ala Leu Ser Ser Tyr Thr Asp Asp Lys Ile	
20 25 30 35	
TTA ATT TCA TAT TTT AAT AAA TTC TTT AAG AGA ATT AAA AGT AGT TCA	260
Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys Ser Ser Ser	
40 45 50	
GTT TTA AAT ATG AGA TAT AAA AAT GAT AAA TAC GTA GAT ACT TCA GGA	308
Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp Thr Ser Gly	
55 60 65	
TAT GAT TCA AAT ATA AAT ATT AAT GGA GAT GTA TAT AAA TAT CCA ACT	356
Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys Tyr Pro Thr	
70 75 80	
AAT AAA AAT CAA TTT GGA ATA TAT AAT GAT AAA CTT AGT GAA GTT AAT	404
Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser Glu Val Asn	
85 90 95	
ATA TCT CAA AAT GAT TAC ATT ATA TAT GAT AAT AAA TAT AAA AAT TTT	452
Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr Lys Asn Phe	
100 105 110 115	
AGT ATT AGT TTT TGG GTA AGA ATT CCT AAC TAT GAT AAT AAG ATA GTA	500
Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn Lys Ile Val	
120 125 130	
AAT GTT AAT AAT GAA TAC ACT ATA ATA AAT TGT ATG AGA GAT AAT AAT	548
Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg Asp Asn Asn	
135 140 145	
TCA GGA TGG AAA GTA TCT CTT AAT CAT AAT GAA ATA ATT TGG ACA TTG	596
Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile Trp Thr Leu	
150 155 160	
CAA GAT AAT GCA GGA ATT AAT CAA AAA TTA GCA TTT AAC TAT GGT AAC	644
Gln Asp Asn Ala Gly Ile Asn Gln Lys Leu Ala Phe Asn Tyr Gly Asn	
165 170 175	
GCA AAT GGT ATT TCT GAT TAT ATA AAT AAG TGG ATT TTT GTA ACT ATA	692
Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile	
180 185 190 195	
ACT AAT GAT AGA TTA GGA GAT TCT AAA CTT TAT ATT AAT GGA AAT TTA	740
Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn Gly Asn Leu	
200 205 210	
ATA GAT CAA AAA TCA ATT TTA AAT TTA GGT AAT ATT CAT GTT AGT GAC	788
Ile Asp Gln Lys Ser Ile Leu Asn Leu Gly Asn Ile His Val Ser Asp	
215 220 225	
AAT ATA TTA TTT AAA ATA GTT AAT TGT AGT TAT ACA AGA TAT ATT GGT	836
Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg Tyr Ile Gly	
230 235 240	
ATT AGA TAT TTT AAT ATT TTT GAT AAA GAA TTA GAT GAA ACA GAA ATT	884
Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu Thr Glu Ile	
245 250 255	

CAA ACT TTA TAT AGC AAT GAA CCT AAT ACA AAT ATT TTG AAG GAT TTT Gln Thr Leu Tyr Ser Asn Glu Pro Asn Thr Asn Ile Leu Lys Asp Phe 260 265 270 275	932
TGG GGA AAT TAT TTG CTT TAT GAC AAA GAA TAC TAT TTA TTA AAT GTG Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu Leu Asn Val 280 285 290	980
TTA AAA CCA AAT AAC TTT ATT GAT AGG AGA AAA GAT TCT ACT TTA AGC Leu Lys Pro Asn Asn Phe Ile Asp Arg Arg Lys Asp Ser Thr Leu Ser 295 300 305	1028
ATT AAT AAT ATA AGA AGC ACT ATT CTT TTA GCT AAT AGA TTA TAT AGT Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg Leu Tyr Ser 310 315 320	1076
GGA ATA AAA GTT AAA ATA CAA AGA GTT AAT AAT AGT AGT ACT AAC GAT Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser Thr Asn Asp 325 330 335	1124
AAT CTT GTT AGA AAG AAT GAT CAG GTA TAT ATT AAT TTT GTA GCC AGC Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe Val Ala Ser 340 345 350 355	1172
AAA ACT CAC TTA TTT CCA TTA TAT GCT GAT ACA GCT ACC ACA AAT AAA Lys Thr His Leu Phe Pro Leu Tyr Ala Asp Thr Ala Thr Thr Asn Lys 360 365 370	1220
GAG AAA ACA ATA AAA ATA TCA TCA TCT GGC AAT AGA TTT AAT CAA GTA Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe Asn Gln Val 375 380 385	1268
GTA GTT ATG AAT TCA GTA GGA AAT AAT TGT ACA ATG AAT TTT AAA AAT Val Val Met Asn Ser Val Gly Asn Asn Cys Thr Met Asn Phe Lys Asn 390 395 400	1316
AAT AAT GGA AAT AAT ATT GGG TTG TTA GGT TTC AAG GCA GAT ACT GTA Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala Asp Thr Val 405 410 415	1364
GTT GCT AGT ACT TGG TAT TAT ACA CAT ATG AGA GAT CAT ACA AAC AGC Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp His Thr Asn Ser 420 425 430 435	1412
AAT GGA TGT TTT TGG AAC TTT ATT TCT GAA GAA CAT GGA TGG CAA GAA Asn Gly Cys Phe Trp Asn Phe Ile Ser Glu Glu His Gly Trp Gln Glu 440 445 450	1460
AAA TAAAAGCTT Lys	1472

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 452 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Met Gly His His His His His His His His His Ser Ser Gly His  
1 5 10 15  
Ile Glu Gly Arg His Met Ala Ser Met Ala Leu Ser Ser Tyr Thr Asp  
20 25 30  
Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys  
35 40 45  
Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp  
50 55 60  
Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys  
65 70 75 80  
Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser  
85 90 95  
Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr  
100 105 110  
Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn  
115 120 125  
Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg  
130 135 140  
Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile  
145 150 155 160  
Trp Thr Leu Gln Asp Asn Ala Gly Ile Asn Gln Lys Leu Ala Phe Asn  
165 170 175  
Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe  
180 185 190  
Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn  
195 200 205  
Gly Asn Leu Ile Asp Gln Lys Ser Ile Leu Asn Leu Gly Asn Ile His  
210 215 220  
Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg  
225 230 235 240  
Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu  
245 250 255  
Thr Glu Ile Gln Thr Leu Tyr Ser Asn Glu Pro Asn Thr Asn Ile Leu  
260 265 270  
Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu  
275 280 285  
Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asp Arg Arg Lys Asp Ser  
290 295 300  
Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg  
305 310 315 320



(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3876 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..3873

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

ATG CCA ATA ACA ATT AAC AAC TTT AAT TAT TCA GAT CCT GTT GAT AAT	48
Met Pro Ile Thr Ile Asn Asn Phe Asn Tyr Ser Asp Pro Val Asp Asn	
1 5 10 15	
AAA AAT ATT TTA TAT TTA GAT ACT CAT TTA AAT ACA CTA GCT AAT GAG	96
Lys Asn Ile Leu Tyr Leu Asp Thr His Leu Asn Thr Leu Ala Asn Glu	
20 25 30	
CCT GAA AAA GCC TTT CGC ATT ACA GGA AAT ATA TGG GTA ATA CCT GAT	144
Pro Glu Lys Ala Phe Arg Ile Thr Gly Asn Ile Trp Val Ile Pro Asp	
35 40 45	
AGA TTT TCA AGA AAT TCT AAT CCA AAT TTA AAT AAA CCT CCT CGA GTT	192
Arg Phe Ser Arg Asn Ser Asn Pro Asn Leu Asn Lys Pro Pro Arg Val	
50 55 60	
ACA AGC CCT AAA AGT GGT TAT TAT GAT CCT AAT TAT TTG AGT ACT GAT	240
Thr Ser Pro Lys Ser Gly Tyr Tyr Asp Pro Asn Tyr Leu Ser Thr Asp	
65 70 75 80	
TCT GAC AAA GAT ACA TTT TTA AAA GAA ATT ATA AAG TTA TTT AAA AGA	288
Ser Asp Lys Asp Thr Phe Leu Lys Glu Ile Ile Lys Leu Phe Lys Arg	
85 90 95	
ATT AAT TCT AGA GAA ATA GGA GAA GAA TTA ATA TAT AGA CTT TCG ACA	336
Ile Asn Ser Arg Glu Ile Gly Glu Glu Leu Ile Tyr Arg Leu Ser Thr	
100 105 110	
GAT ATA CCC TTT CCT GGG AAT AAC AAT ACT CCA ATT AAT ACT TTT GAT	384
Asp Ile Pro Phe Pro Gly Asn Asn Asn Thr Pro Ile Asn Thr Phe Asp	
115 120 125	
TTT GAT GTA GAT TTT AAC AGT GTT GAT GTT AAA ACT AGA CAA GGT AAC	432
Phe Asp Val Asp Phe Asn Ser Val Asp Val Lys Thr Arg Gln Gly Asn	
130 135 140	
AAC TGG GTT AAA ACT GGT AGC ATA AAT CCT AGT GTT ATA ATA ACT GGA	480
Asn Trp Val Lys Thr Gly Ser Ile Asn Pro Ser Val Ile Ile Thr Gly	
145 150 155 160	
CCT AGA GAA AAC ATT ATA GAT CCA GAA ACT TCT ACG TTT AAA TTA ACT	528
Pro Arg Glu Asn Ile Ile Asp Pro Glu Thr Ser Thr Phe Lys Leu Thr	
165 170 175	

AAC AAT ACT TTT GCG GCA CAA GAA GGA TTT GGT GCT TTA TCA ATA ATT Asn Asn Thr Phe Ala Ala Gln Glu Gly Phe Gly Ala Leu Ser Ile Ile 180 185 190	576
TCA ATA TCA CCT AGA TTT ATG CTA ACA TAT AGT AAT GCA ACT AAT GAT Ser Ile Ser Pro Arg Phe Met Leu Thr Tyr Ser Asn Ala Thr Asn Asp 195 200 205	624
GTA GGA GAG GGT AGA TTT TCT AAG TCT GAA TTT TGC ATG GAT CCA ATA Val Gly Glu Gly Arg Phe Ser Lys Ser Glu Phe Cys Met Asp Pro Ile 210 215 220	672
CTA ATT TTA ATG CAT GAA CTT AAT CAT GCA ATG CAT AAT TTA TAT GGA Leu Ile Leu Met His Glu Leu Asn His Ala Met His Asn Leu Tyr Gly 225 230 235 240	720
ATA GCT ATA CCA AAT GAT CAA ACA ATT TCA TCT GTA ACT AGT AAT ATT Ile Ala Ile Pro Asn Asp Gln Thr Ile Ser Ser Val Thr Ser Asn Ile 245 250 255	768
TTT TAT TCT CAA TAT AAT GTG AAA TTA GAG TAT GCA GAA ATA TAT GCA Phe Tyr Ser Gln Tyr Asn Val Lys Leu Glu Tyr Ala Glu Ile Tyr Ala 260 265 270	816
TTT GGA GGT CCA ACT ATA GAC CTT ATT CCT AAA AGT GCA AGG AAA TAT Phe Gly Gly Pro Thr Ile Asp Leu Ile Pro Lys Ser Ala Arg Lys Tyr 275 280 285	864
TTT GAG GAA AAG GCA TTG GAT TAT TAT AGA TCT ATA GCT AAA AGA CTT Phe Glu Glu Lys Ala Leu Asp Tyr Tyr Arg Ser Ile Ala Lys Arg Leu 290 295 300	912
AAT AGT ATA ACT ACT GCA AAT CCT TCA AGC TTT AAT AAA TAT ATA GGG Asn Ser Ile Thr Thr Ala Asn Pro Ser Ser Phe Asn Lys Tyr Ile Gly 305 310 315 320	960
GAA TAT AAA CAG AAA CTT ATT AGA AAG TAT AGA TTC GTA GTA GAA TCT Glu Tyr Lys Gln Lys Leu Ile Arg Lys Tyr Arg Phe Val Val Glu Ser 325 330 335	1008
TCA GGT GAA GTT ACA GTA AAT CGT AAT AAG TTT GTT GAG TTA TAT AAT Ser Gly Glu Val Thr Val Asn Arg Asn Lys Phe Val Glu Leu Tyr Asn 340 345 350	1056
GAA CTT ACA CAA ATA TTT ACA GAA TTT AAC TAC GCT AAA ATA TAT AAT Glu Leu Thr Gln Ile Phe Thr Glu Phe Asn Tyr Ala Lys Ile Tyr Asn 355 360 365	1104
GTA CAA AAT AGG AAA ATA TAT CTT TCA AAT GTA TAT ACT CCG GTT ACG Val Gln Asn Arg Lys Ile Tyr Leu Ser Asn Val Tyr Thr Pro Val Thr 370 375 380	1152
GCG AAT ATA TTA GAC GAT AAT GTT TAT GAT ATA CAA AAT GGA TTT AAT Ala Asn Ile Leu Asp Asp Asn Val Tyr Asp Ile Gln Asn Gly Phe Asn 385 390 395 400	1200
ATA CCT AAA AGT AAT TTA AAT GTA CTA TTT ATG GGT CAA AAT TTA TCT Ile Pro Lys Ser Asn Leu Asn Val Leu Phe Met Gly Gln Asn Leu Ser 405 410 415	1248

CGA AAT CCA GCA TTA AGA AAA GTC AAT CCT GAA AAT ATG CTT TAT TTA Arg Asn Pro Ala Leu Arg Lys Val Asn Pro Glu Asn Met Leu Tyr Leu 420 425 430	1296
TTT ACA AAA TTT TGT CAT AAA GCA ATA GAT GGT AGA TCA TTA TAT AAT Phe Thr Lys Phe Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn 435 440 445	1344
AAA ACA TTA GAT TGT AGA GAG CTT TTA GTT AAA AAT ACT GAC TTA CCC Lys Thr Leu Asp Cys Arg Glu Leu Val Lys Asn Thr Asp Leu Pro 450 455 460	1392
TTT ATA GGT GAT ATT AGT GAT GTT AAA ACT GAT ATA TTT TTA AGA AAA Phe Ile Gly Asp Ile Ser Asp Val Lys Thr Asp Ile Phe Leu Arg Lys 465 470 475 480	1440
GAT ATT AAT GAA GAA ACT GAA GTT ATA TAC TAT CCG GAC AAT GTT TCA Asp Ile Asn Glu Glu Thr Glu Val Ile Tyr Tyr Pro Asp Asn Val Ser 485 490 495	1488
GTA GAT CAA GTT ATT CTC AGT AAG AAT ACC TCA GAA CAT GGA CAA CTA Val Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu His Gly Gln Leu 500 505 510	1536
GAT TTA TTA TAC CCT AGT ATT GAC AGT GAG AGT GAA ATA TTA CCA GGG Asp Leu Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu Ile Leu Pro Gly 515 520 525	1584
GAG AAT CAA GTC TTT TAT GAT AAT AGA ACT CAA AAT GTT GAT TAT TTG Glu Asn Gln Val Phe Tyr Asp Asn Arg Thr Gln Asn Val Asp Tyr Leu 530 535 540	1632
AAT TCT TAT TAT TAC CTA GAA TCT CAA AAA CTA AGT GAT AAT GTT GAA Asn Ser Tyr Tyr Tyr Leu Glu Ser Gln Lys Leu Ser Asp Asn Val Glu 545 550 555 560	1680
GAT TTT ACT TTT ACG AGA TCA ATT GAG GAG GCT TTG GAT AAT AGT GCA Asp Phe Thr Phe Thr Arg Ser Ile Glu Glu Ala Leu Asp Asn Ser Ala 565 570 575	1728
AAA GTA TAT ACT TAC TTT CCT ACA CTA GCT AAT AAA GTA AAT GCG GGT Lys Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys Val Asn Ala Gly 580 585 590	1776
GTT CAA GGT GGT TTA TTT TTA ATG TGG GCA AAT GAT GTA GTT GAA GAT Val Gln Gly Gly Leu Phe Leu Met Trp Ala Asn Asp Val Val Glu Asp 595 600 605	1824
TTT ACT ACA AAT ATT CTA AGA AAA GAT ACA TTA GAT AAA ATA TCA GAT Phe Thr Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp Lys Ile Ser Asp 610 615 620	1872
GTA TCA GCT ATT ATT CCC TAT ATA GGA CCC GCA TTA AAT ATA AGT AAT Val Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Ser Asn 625 630 635 640	1920
TCT GTA AGA AGA GGA AAT TTT ACT GAA GCA TTT GCA GTT ACT GGT GTA Ser Val Arg Arg Gly Asn Phe Thr Glu Ala Phe Ala Val Thr Gly Val 645 650 655	1968

ACT ATT TTA TTA GAA GCA TTT CCT GAA TTT ACA ATA CCT GCA CTT GGT Thr Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly 660 665 670	2016
GCA TTT GTG ATT TAT AGT AAG GTT CAA GAA AGA AAC GAG ATT ATT AAA Ala Phe Val Ile Tyr Ser Lys Val Gln Glu Arg Asn Glu Ile Ile Lys 675 680 685	2064
ACT ATA GAT AAT TGT TTA GAA CAA AGG ATT AAG AGA TGG AAA GAT TCA Thr Ile Asp Asn Cys Leu Glu Gln Arg Ile Lys Arg Trp Lys Asp Ser 690 695 700	2112
TAT GAA TGG ATG ATG GGA ACG TGG TTA TCC AGG ATT ATT ACT CAA TTT Tyr Glu Trp Met Met Gly Thr Trp Leu Ser Arg Ile Ile Thr Gln Phe 705 710 715 720	2160
AAT AAT ATA AGT TAT CAA ATG TAT GAT TCT TTA AAT TAT CAG GCA GGT Asn Asn Ile Ser Tyr Gln Met Tyr Asp Ser Leu Asn Tyr Gln Ala Gly 725 730 735	2208
GCA ATC AAA GCT AAA ATA GAT TTA GAA TAT AAA AAA TAT TCA GGA AGT Ala Ile Lys Ala Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser 740 745 750	2256
GAT AAA GAA AAT ATA AAA AGT CAA GTT GAA AAT TTA AAA AAT AGT TTA Asp Lys Glu Asn Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu 755 760 765	2304
GAT GTA AAA ATT TCG GAA GCA ATG AAT AAT ATA AAT AAA TTT ATA CGA Asp Val Lys Ile Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg 770 775 780	2352
GAA TGT TCC GTA ACA TAT TTA TTT AAA AAT ATG TTA CCT AAA GTA ATT Glu Cys Ser Val Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile 785 790 795 800	2400
GAT GAA TTA AAT GAG TTT GAT CGA AAT ACT AAA GCA AAA TTA ATT AAT Asp Glu Leu Asn Glu Phe Asp Arg Asn Thr Lys Ala Lys Leu Ile Asn 805 810 815	2448
CTT ATA GAT AGT CAT AAT ATT ATT CTA GTT GGT GAA GTA GAT AAA TTA Leu Ile Asp Ser His Asn Ile Ile Leu Val Gly Glu Val Asp Lys Leu 820 825 830	2496
AAA GCA AAA GTA AAT AAT AGC TTT CAA AAT ACA ATA CCC TTT AAT ATT Lys Ala Lys Val Asn Asn Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile 835 840 845	2544
TTT TCA TAT ACT AAT AAT TCT TTA TTA AAA GAT ATA ATT AAT GAA TAT Phe Ser Tyr Thr Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr 850 855 860	2592
TTC AAT AAT ATT AAT GAT TCA AAA ATT TTG AGC CTA CAA AAC AGA AAA Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys 865 870 875 880	2640
AAT ACT TTA GTG GAT ACA TCA GGA TAT AAT GCA GAA GTG AGT GAA GAA Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu 885 890 895	2688

GGC GAT GTT CAG CTT AAT CCA ATA TTT CCA TTT GAC TTT AAA TTA GGT Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly 900 905 910	2736
AGT TCA GGG GAG GAT AGA GGT AAA GTT ATA GTA ACC CAG AAT GAA AAT Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn 915 920 925	2784
ATT GTA TAT AAT TCT ATG TAT GAA AGT TTT AGC ATT AGT TTT TGG ATT Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile 930 935 940	2832
AGA ATA AAT AAA TGG GTA AGT AAT TTA CCT GGA TAT ACT ATA ATT GAT Arg Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp 945 950 955 960	2880
AGT GTT AAA AAT AAC TCA GGT TGG AGT ATA GGT ATT ATT AGT AAT TTT Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe 965 970 975	2928
TTA GTA TTT ACT TTA AAA CAA AAT GAA GAT AGT GAA CAA AGT ATA AAT Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn 980 985 990	2976
TTT AGT TAT GAT ATA TCA AAT AAT GCT CCT GGA TAC AAT AAA TGG TTT Phe Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe 995 1000 1005	3024
TTT GTA ACT GTT ACT AAC AAT ATG ATG GGA AAT ATG AAG ATT TAT ATA Phe Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile 1010 1015 1020	3072
AAT GGA AAA TTA ATA GAT ACT ATA AAA GTT AAA GAA CTA ACT GGA ATT Asn Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile 1025 1030 1035 1040	3120
AAT TTT AGC AAA ACT ATA ACA TTT GAA ATA AAT AAA ATT CCA GAT ACC Asn Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr 1045 1050 1055	3168
GGT TTG ATT ACT TCA GAT TCT GAT AAC ATC AAT ATG TGG ATA AGA GAT Gly Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp 1060 1065 1070	3216
TTT TAT ATA TTT GCT AAA GAA TTA GAT GGT AAA GAT ATT AAT ATA TTA Phe Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu 1075 1080 1085	3264
TTT AAT AGC TTG CAA TAT ACT AAT GTT GTA AAA GAT TAT TGG GGA AAT Phe Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn 1090 1095 1100	3312
GAT TTA AGA TAT AAT AAA GAA TAT TAT ATG GTT AAT ATA GAT TAT TTA Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu 1105 1110 1115 1120	3360
AAT AGA TAT ATG TAT GCG AAC TCA CGA CAA ATT GTT TTT AAT ACA CGT Asn Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg 1125 1130 1135	3408

AGA AAT AAT AAT GAC TTC AAT GAA GGA TAT AAA ATT ATA ATA AAA AGA Arg Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg 1140 1145 1150	3456
ATC AGA GGA AAT ACA AAT GAT ACT AGA GTA CGA GGA GGA GAT ATT TTA Ile Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu 1155 1160 1165	3504
TAT TTT GAT ATG ACA ATT AAT AAC AAA GCA TAT AAT TTG TTT ATG AAG Tyr Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys 1170 1175 1180	3552
AAT GAA ACT ATG TAT GCA GAT AAT CAT AGT ACT GAA GAT ATA TAT GCT Asn Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala 1185 1190 1195 1200	3600
ATA GGT TTA AGA GAA CAA ACA AAG GAT ATA AAT GAT AAT ATT ATA TTT Ile Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe 1205 1210 1215	3648
CAA ATA CAA CCA ATG AAT AAT ACT TAT TAT TAC GCA TCT CAA ATA TTT Gln Ile Gln Pro Met Asn Asn Thr Tyr Tyr Ala Ser Gln Ile Phe 1220 1225 1230	3696
AAA TCA AAT TTT AAT GGA GAA AAT ATT TCT GGA ATA TGT TCA ATA GGT Lys Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly 1235 1240 1245	3744
ACT TAT CGT TTT AGA CTT GGA GGT GAT TGG TAT AGA CAC AAT TAT TTG Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr Leu 1250 1255 1260	3792
GTG CCT ACT GTG AAG CAA GGA AAT TAT GCT TCA TTA TTA GAA TCA ACA Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu Glu Ser Thr 1265 1270 1275 1280	3840
TCA ACT CAT TGG GGT TTT GTA CCT GTA AGT GAA TAA Ser Thr His Trp Gly Phe Val Pro Val Ser Glu 1285 1290	3876

(2) INFORMATION FOR SEQ ID NO:60:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1291 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Met Pro Ile Thr Ile Asn Asn Phe Asn Tyr Ser Asp Pro Val Asp Asn 1 5 10 15
Lys Asn Ile Leu Tyr Leu Asp Thr His Leu Asn Thr Leu Ala Asn Glu 20 25 30
Pro Glu Lys Ala Phe Arg Ile Thr Gly Asn Ile Trp Val Ile Pro Asp 35 40 45
Arg Phe Ser Arg Asn Ser Asn Pro Asn Leu Asn Lys Pro Pro Arg Val 50 55 60



Thr Ser Pro Lys Ser Gly Tyr Tyr Asp Pro Asn Tyr Leu Ser Thr Asp  
 65 70 75 80  
 Ser Asp Lys Asp Thr Phe Leu Lys Glu Ile Ile Lys Leu Phe Lys Arg  
 85 90 95  
 Ile Asn Ser Arg Glu Ile Gly Glu Glu Leu Ile Tyr Arg Leu Ser Thr  
 100 105 110  
 Asp Ile Pro Phe Pro Gly Asn Asn Asn Thr Pro Ile Asn Thr Phe Asp  
 115 120 125  
 Phe Asp Val Asp Phe Asn Ser Val Asp Val Lys Thr Arg Gln Gly Asn  
 130 135 140  
 Asn Trp Val Lys Thr Gly Ser Ile Asn Pro Ser Val Ile Ile Thr Gly  
 145 150 155 160  
 Pro Arg Glu Asn Ile Ile Asp Pro Glu Thr Ser Thr Phe Lys Leu Thr  
 165 170 175  
 Asn Asn Thr Phe Ala Ala Gln Glu Gly Phe Gly Ala Leu Ser Ile Ile  
 180 185 190  
 Ser Ile Ser Pro Arg Phe Met Leu Thr Tyr Ser Asn Ala Thr Asn Asp  
 195 200 205  
 Val Gly Glu Gly Arg Phe Ser Lys Ser Glu Phe Cys Met Asp Pro Ile  
 210 215 220  
 Leu Ile Leu Met His Glu Leu Asn His Ala Met His Asn Leu Tyr Gly  
 225 230 235 240  
 Ile Ala Ile Pro Asn Asp Gln Thr Ile Ser Ser Val Thr Ser Asn Ile  
 245 250 255  
 Phe Tyr Ser Gln Tyr Asn Val Lys Leu Glu Tyr Ala Glu Ile Tyr Ala  
 260 265 270  
 Phe Gly Gly Pro Thr Ile Asp Leu Ile Pro Lys Ser Ala Arg Lys Tyr  
 275 280 285  
 Phe Glu Glu Lys Ala Leu Asp Tyr Tyr Arg Ser Ile Ala Lys Arg Leu  
 290 295 300  
 Asn Ser Ile Thr Thr Ala Asn Pro Ser Ser Phe Asn Lys Tyr Ile Gly  
 305 310 315 320  
 Glu Tyr Lys Gln Lys Leu Ile Arg Lys Tyr Arg Phe Val Val Glu Ser  
 325 330 335  
 Ser Gly Glu Val Thr Val Asn Arg Asn Lys Phe Val Glu Leu Tyr Asn  
 340 345 350  
 Glu Leu Thr Gln Ile Phe Thr Glu Phe Asn Tyr Ala Lys Ile Tyr Asn  
 355 360 365  
 Val Gln Asn Arg Lys Ile Tyr Leu Ser Asn Val Tyr Thr Pro Val Thr  
 370 375 380  
 Ala Asn Ile Leu Asp Asp Asn Val Tyr Asp Ile Gln Asn Gly Phe Asn  
 385 390 395 400

Ile Pro Lys Ser Asn Leu Asn Val Leu Phe Met Gly Gln Asn Leu Ser  
 405 410 415  
 Arg Asn Pro Ala Leu Arg Lys Val Asn Pro Glu Asn Met Leu Tyr Leu  
 420 425 430  
 Phe Thr Lys Phe Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn  
 435 440 445  
 Lys Thr Leu Asp Cys Arg Glu Leu Leu Val Lys Asn Thr Asp Leu Pro  
 450 455 460  
 Phe Ile Gly Asp Ile Ser Asp Val Lys Thr Asp Ile Phe Leu Arg Lys  
 465 470 475 480  
 Asp Ile Asn Glu Glu Thr Glu Val Ile Tyr Tyr Pro Asp Asn Val Ser  
 485 490 495  
 Val Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu His Gly Gln Leu  
 500 505 510  
 Asp Leu Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu Ile Leu Pro Gly  
 515 520 525  
 Glu Asn Gln Val Phe Tyr Asp Asn Arg Thr Gln Asn Val Asp Tyr Leu  
 530 535 540  
 Asn Ser Tyr Tyr Tyr Leu Glu Ser Gln Lys Leu Ser Asp Asn Val Glu  
 545 550 555 560  
 Asp Phe Thr Phe Thr Arg Ser Ile Glu Glu Ala Leu Asp Asn Ser Ala  
 565 570 575  
 Lys Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys Val Asn Ala Gly  
 580 585 590  
 Val Gln Gly Gly Leu Phe Leu Met Trp Ala Asn Asp Val Val Glu Asp  
 595 600 605  
 Phe Thr Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp Lys Ile Ser Asp  
 610 615 620  
 Val Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Ser Asn  
 625 630 635 640  
 Ser Val Arg Arg Gly Asn Phe Thr Glu Ala Phe Ala Val Thr Gly Val  
 645 650 655  
 Thr Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly  
 660 665 670  
 Ala Phe Val Ile Tyr Ser Lys Val Gln Glu Arg Asn Glu Ile Ile Lys  
 675 680 685  
 Thr Ile Asp Asn Cys Leu Glu Gln Arg Ile Lys Arg Trp Lys Asp Ser  
 690 695 700  
 Tyr Glu Trp Met Met Gly Thr Trp Leu Ser Arg Ile Ile Thr Gln Phe  
 705 710 715 720  
 Asn Asn Ile Ser Tyr Gln Met Tyr Asp Ser Leu Asn Tyr Gln Ala Gly  
 725 730 735

Ala Ile Lys Ala Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser  
 740 745 750  
 Asp Lys Glu Asn Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu  
 755 760 765  
 Asp Val Lys Ile Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg  
 770 775 780  
 Glu Cys Ser Val Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile  
 785 790 795 800  
 Asp Glu Leu Asn Glu Phe Asp Arg Asn Thr Lys Ala Lys Leu Ile Asn  
 805 810 815  
 Leu Ile Asp Ser His Asn Ile Ile Leu Val Gly Glu Val Asp Lys Leu  
 820 825 830  
 Lys Ala Lys Val Asn Asn Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile  
 835 840 845  
 Phe Ser Tyr Thr Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr  
 850 855 860  
 Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys  
 865 870 875 880  
 Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu  
 885 890 895  
 Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly  
 900 905 910  
 Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn  
 915 920 925  
 Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile  
 930 935 940  
 Arg Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp  
 945 950 955 960  
 Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe  
 965 970 975  
 Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn  
 980 985 990  
 Phe Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe  
 995 1000 1005  
 Phe Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile  
 1010 1015 1020  
 Asn Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile  
 1025 1030 1035 1040  
 Asn Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr  
 1045 1050 1055  
 Gly Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp  
 1060 1065 1070

Phe Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu  
 1075 1080 1085  
 Phe Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn  
 1090 1095 1100  
 Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu  
 1105 1110 1115 1120  
 Asn Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg  
 1125 1130 1135  
 Arg Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg  
 1140 1145 1150  
 Ile Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu  
 1155 1160 1165  
 Tyr Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys  
 1170 1175 1180  
 Asn Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala  
 1185 1190 1195 1200  
 Ile Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe  
 1205 1210 1215  
 Gln Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe  
 1220 1225 1230  
 Lys Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly  
 1235 1240 1245  
 Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr Leu  
 1250 1255 1260  
 Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu Glu Ser Thr  
 1265 1270 1275 1280  
 Ser Thr His Trp Gly Phe Val Pro Val Ser Glu  
 1285 1290

(2) INFORMATION FOR SEQ ID NO:61:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1502 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 108..1493

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATAACC ATG GGC CAT	116
Met Gly His	
1	
CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT	164
His His His His His His His His His Ser Ser Gly His Ile Glu Gly	
5 10 15	
CGT CAT ATG GCT AGC ATG GCT TTA TTA AAA GAT ATA ATT AAT GAA TAT	212
Arg His Met Ala Ser Met Ala Leu Leu Lys Asp Ile Ile Asn Glu Tyr	
20 25 30 35	
TTC AAT AAT ATT AAT GAT TCA AAA ATT TTG AGC CTA CAA AAC AGA AAA	260
Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys	
40 45 50	
AAT ACT TTA GTG GAT ACA TCA GGA TAT AAT GCA GAA GTG AGT GAA GAA	308
Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu	
55 60 65	
GGC GAT GTT CAG CTT AAT CCA ATA TTT CCA TTT GAC TTT AAA TTA GGT	356
Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly	
70 75 80	
AGT TCA GGG GAG GAT AGA GGT AAA GTT ATA GTA ACC CAG AAT GAA AAT	404
Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn	
85 90 95	
ATT GTA TAT AAT TCT ATG TAT GAA AGT TTT AGC ATT AGT TTT TGG ATT	452
Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile	
100 105 110 115	
AGA ATA AAT AAA TGG GTA AGT AAT TTA CCT GGA TAT ACT ATA ATT GAT	500
Arg Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp	
120 125 130	
AGT GTT AAA AAT AAC TCA GGT TGG AGT ATA GGT ATT ATT AGT AAT TTT	548
Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe	
135 140 145	
TTA GTA TTT ACT TTA AAA CAA AAT GAA GAT AGT GAA CAA AGT ATA AAT	596
Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn	
150 155 160	
TTT AGT TAT GAT ATA TCA AAT AAT GCT CCT GGA TAC AAT AAA TGG TTT	644
Phe Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe	
165 170 175	
TTT GTA ACT GTT ACT AAC AAT ATG ATG GGA AAT ATG AAG ATT TAT ATA	692
Phe Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile	
180 185 190 195	
AAT GGA AAA TTA ATA GAT ACT ATA AAA GTT AAA GAA CTA ACT GGA ATT	740
Asn Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile	
200 205 210	

AAT TTT AGC AAA ACT ATA ACA TTT GAA ATA AAT AAA ATT CCA GAT ACC Asn Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr 215 220 225	788
GGT TTG ATT ACT TCA GAT TCT GAT AAC ATC AAT ATG TGG ATA AGA GAT Gly Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp 230 235 240	836
TTT TAT ATA TTT GCT AAA GAA TTA GAT GGT AAA GAT ATT AAT ATA TTA Phe Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu 245 250 255	884
TTT AAT AGC TTG CAA TAT ACT AAT GTT GTA AAA GAT TAT TGG GGA AAT Phe Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn 260 265 270 275	932
GAT TTA AGA TAT AAT AAA GAA TAT TAT ATG GTT AAT ATA GAT TAT TTA Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu 280 285 290	980
AAT AGA TAT ATG TAT GCG AAC TCA CGA CAA ATT GTT TTT AAT ACA CGT Asn Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg 295 300 305	1028
AGA AAT AAT AAT GAC TTC AAT GAA GGA TAT AAA ATT ATA ATA AAA AGA Arg Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg 310 315 320	1076
ATC AGA GGA AAT ACA AAT GAT ACT AGA GTA CGA GGA GGA GAT ATT TTA Ile Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu 325 330 335	1124
TAT TTT GAT ATG ACA ATT AAT AAC AAA GCA TAT AAT TTG TTT ATG AAG Tyr Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys 340 345 350 355	1172
AAT GAA ACT ATG TAT GCA GAT AAT CAT AGT ACT GAA GAT ATA TAT GCT Asn Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala 360 365 370	1220
ATA GGT TTA AGA GAA CAA ACA AAG GAT ATA AAT GAT AAT ATT ATA TTT Ile Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe 375 380 385	1268
CAA ATA CAA CCA ATG AAT AAT ACT TAT TAT TAC GCA TCT CAA ATA TTT Gln Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe 390 395 400	1316
AAA TCA AAT TTT AAT GGA GAA AAT ATT TCT GGA ATA TGT TCA ATA GGT Lys Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly 405 410 415	1364
ACT TAT CGT TTT AGA CTT GGA GGT GAT TGG TAT AGA CAC AAT TAT TTG Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr Leu 420 425 430 435	1412
GTG CCT ACT GTG AAG CAA GGA AAT TAT GCT TCA TTA TTA GAA TCA ACA Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu Glu Ser Thr 440 445 450	1460

TCA ACT CAT TGG GGT TTT GTA CCT GTA AGT GAA TAAAAGCTT  
 Ser Thr His Trp Gly Phe Val Pro Val Ser Glu  
 455 460

1502

(2) INFORMATION FOR SEQ ID NO:62:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 462 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Met Gly His His His His His His His His His His His Ser Ser Gly His  
 1 5 10 15  
 Ile Glu Gly Arg His Met Ala Ser Met Ala Leu Leu Lys Asp Ile Ile  
 20 25 30  
 Asn Glu Tyr Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln  
 35 40 45  
 Asn Arg Lys Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val  
 50 55 60  
 Ser Glu Glu Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe  
 65 70 75 80  
 Lys Leu Gly Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln  
 85 90 95  
 Asn Glu Asn Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser  
 100 105 110  
 Phe Trp Ile Arg Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr  
 115 120 125  
 Ile Ile Asp Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile  
 130 135 140  
 Ser Asn Phe Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln  
 145 150 155 160  
 Ser Ile Asn Phe Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn  
 165 170 175  
 Lys Trp Phe Phe Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys  
 180 185 190  
 Ile Tyr Ile Asn Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu  
 195 200 205  
 Thr Gly Ile Asn Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile  
 210 215 220  
 Pro Asp Thr Gly Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp  
 225 230 235 240  
 Ile Arg Asp Phe Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile  
 245 250 255

Asn Ile Leu Phe Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr  
 260 265 270  
 Trp Gly Asn Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile  
 275 280 285  
 Asp Tyr Leu Asn Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe  
 290 295 300  
 Asn Thr Arg Arg Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile  
 305 310 315 320  
 Ile Lys Arg Ile Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly  
 325 330 335  
 Asp Ile Leu Tyr Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu  
 340 345 350  
 Phe Met Lys Asn Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp  
 355 360 365  
 Ile Tyr Ala Ile Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn  
 370 375 380  
 Ile Ile Phe Gln Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser  
 385 390 395 400  
 Gln Ile Phe Lys Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys  
 405 410 415  
 Ser Ile Gly Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His  
 420 425 430  
 Asn Tyr Leu Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu  
 435 440 445  
 Glu Ser Thr Ser Thr His Trp Gly Phe Val Pro Val Ser Glu  
 450 455 460

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 32 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

CGCCATGGCT TTATTAAAAG ATATAATTAA TG

32



(2) INFORMATION FOR SEQ ID NO:64:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GCAAGCTTTT ATTCACTTAC AGGTACAAAA CC

32

(2) INFORMATION FOR SEQ ID NO:65:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 3831 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 1..3828

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

ATG ACA TGG CCA GTA AAA GAT TTT AAT TAT AGT GAT CCT GTT AAT GAC	48
Met Thr Trp Pro Val Lys Asp Phe Asn Tyr Ser Asp Pro Val Asn Asp	
1 5 10 15	
AAT GAT ATA TTA TAT TTA AGA ATA CCA CAA AAT AAG TTA ATT ACT ACA	96
Asn Asp Ile Leu Tyr Leu Arg Ile Pro Gln Asn Lys Leu Ile Thr Thr	
20 25 30	
CCT GTA AAA GCT TTT ATG ATT ACT CAA AAT ATT TGG GTA ATA CCA GAA	144
Pro Val Lys Ala Phe Met Ile Thr Gln Asn Ile Trp Val Ile Pro Glu	
35 40 45	
AGA TTT TCA TCA GAT ACT AAT CCA AGT TTA AGT AAA CCG CCC AGA CCT	192
Arg Phe Ser Ser Asp Thr Asn Pro Ser Leu Ser Lys Pro Pro Arg Pro	
50 55 60	
ACT TCA AAG TAT CAA AGT TAT TAT GAT CCT AGT TAT TTA TCT ACT GAT	240
Thr Ser Lys Tyr Gln Ser Tyr Tyr Asp Pro Ser Tyr Leu Ser Thr Asp	
65 70 75 80	
GAA CAA AAA GAT ACA TTT TTA AAA GGG ATT ATA AAA TTA TTT AAA AGA	288
Glu Gln Lys Asp Thr Phe Leu Lys Gly Ile Ile Lys Leu Phe Lys Arg	
85 90 95	
ATT AAT GAA AGA GAT ATA GGA AAA AAA TTA ATA AAT TAT TTA GTA GTT	336
Ile Asn Glu Arg Asp Ile Gly Lys Lys Leu Ile Asn Tyr Leu Val Val	
100 105 110	

GGT TCA CCT TTT ATG GGA GAT TCA AGT ACG CCT GAA GAT ACA TTT GAT Gly Ser Pro Phe Met Gly Asp Ser Ser Thr Pro Glu Asp Thr Phe Asp 115 120 125	384
TTT ACA CGT CAT ACT ACT AAT ATT GCA GTT GAA AAG TTT GAA AAT GGT Phe Thr Arg His Thr Thr Asn Ile Ala Val Glu Lys Phe Glu Asn Gly 130 135 140	432
AGT TGG AAA GTA ACA AAT ATT ATA ACA CCA AGT GTA TTG ATA TTT GGA Ser Trp Lys Val Thr Asn Ile Ile Thr Pro Ser Val Leu Ile Phe Gly 145 150 155 160	480
CCA CTT CCT AAT ATA TTA GAC TAT ACA GCA TCC CTT ACA TTG CAA GGA Pro Leu Pro Asn Ile Leu Asp Tyr Thr Ala Ser Leu Thr Leu Gln Gly 165 170 175	528
CAA CAA TCA AAT CCA TCA TTT GAA GGG TTT GGA ACA TTA TCT ATA CTA Gln Gln Ser Asn Pro Ser Phe Glu Gly Phe Gly Thr Leu Ser Ile Leu 180 185 190	576
AAA GTA GCA CCT GAA TTT TTG TTA ACA TTT AGT GAT GTA ACA TCT AAT Lys Val Ala Pro Glu Phe Leu Leu Thr Phe Ser Asp Val Thr Ser Asn 195 200 205	624
CAA AGT TCA GCT GTA TTA GGC AAA TCT ATA TTT TGT ATG GAT CCA GTA Gln Ser Ser Ala Val Leu Gly Lys Ser Ile Phe Cys Met Asp Pro Val 210 215 220	672
ATA GCT TTA ATG CAT GAG TTA ACA CAT TCT TTG CAT CAA TTA TAT GGA Ile Ala Leu Met His Glu Leu Thr His Ser Leu His Gln Leu Tyr Gly 225 230 235 240	720
ATA AAT ATA CCA TCT GAT AAA AGG ATT CGT CCA CAA GTT AGC GAG GGA Ile Asn Ile Pro Ser Asp Lys Arg Ile Arg Pro Gln Val Ser Glu Gly 245 250 255	768
TTT TTC TCT CAA GAT GGA CCC AAC GTA CAA TTT GAG GAA TTA TAT ACA Phe Phe Ser Gln Asp Gly Pro Asn Val Gln Phe Glu Glu Leu Tyr Thr 260 265 270	816
TTT GGA GGA TTA GAT GTT GAA ATA ATA CCT CAA ATT GAA AGA TCA CAA Phe Gly Gly Leu Asp Val Glu Ile Ile Pro Gln Ile Glu Arg Ser Gln 275 280 285	864
TTA AGA GAA AAA GCA TTA GGT CAC TAT AAA GAT ATA GCG AAA AGA CTT Leu Arg Glu Lys Ala Leu Gly His Tyr Lys Asp Ile Ala Lys Arg Leu 290 295 300	912
AAT AAT ATT AAT AAA ACT ATT CCT TCT AGT TGG ATT AGT AAT ATA GAT Asn Asn Ile Asn Lys Thr Ile Pro Ser Ser Trp Ile Ser Asn Ile Asp 305 310 315 320	960
AAA TAT AAA AAA ATA TTT TCT GAA AAG TAT AAT TTT GAT AAA GAT AAT Lys Tyr Lys Lys Ile Phe Ser Glu Lys Tyr Asn Phe Asp Lys Asp Asn 325 330 335	1008
ACA GGA AAT TTT GTT GTA AAT ATT GAT AAA TTC AAT AGC TTA TAT TCA Thr Gly Asn Phe Val Val Asn Ile Asp Lys Phe Asn Ser Leu Tyr Ser 340 345 350	1056

GAC Asp	TTG Leu	ACT Thr	AAT Asn	GTT Val	ATG Met	TCA Ser	GAA Glu	GTT Val	GTT Val	TAT Tyr	TCT Ser	TCG Ser	CAA Gln	TAT Tyr	AAT Asn	1104
	355						360					365				
GTT Val	AAA Lys	AAC Asn	AGG Arg	ACT Thr	CAT His	TAT Tyr	TTT Phe	TCA Ser	AGG Arg	CAT His	TAT Tyr	CTA Leu	CCT Pro	GTA Val	TTT Phe	1152
	370						375					380				
GCA Ala	AAT Asn	ATA Ile	TTA Leu	GAT Asp	GAT Asp	AAT Asn	ATT Ile	TAT Tyr	ACT Thr	ATA Ile	AGA Arg	GAT Asp	GGT Gly	TTT Phe	AAT Asn	1200
	385						390				395				400	
TTA Leu	ACA Thr	AAT Asn	AAA Lys	GGT Gly	TTT Phe	AAT Asn	ATA Ile	GAA Glu	AAT Asn	TCG Ser	GGT Gly	CAG Gln	AAT Asn	ATA Ile	GAA Glu	1248
				405					410					415		
AGG Arg	AAT Asn	CCT Pro	GCA Ala	CTA Leu	CAA Gln	AAG Lys	CTT Leu	AGT Ser	TCA Ser	GAA Glu	AGT Ser	GTA Val	GTA Val	GAT Asp	TTA Leu	1296
			420					425					430			
TTT Phe	ACA Thr	AAA Lys	GTA Val	TGT Cys	TTA Leu	AGA Arg	TTA Leu	ACA Thr	AAA Lys	AAT Asn	AGT Ser	AGA Arg	GAT Asp	GAT Asp	TCA Ser	1344
		435					440					445				
ACA Thr	TGT Cys	ATT Ile	AAA Lys	GTT Val	AAA Lys	AAT Asn	AAT Asn	AGA Arg	TTA Leu	CCT Pro	TAT Tyr	GTA Val	GCT Ala	GAT Asp	AAA Lys	1392
	450					455					460					
GAT Asp	AGC Ser	ATT Ile	TCA Ser	CAA Gln	GAA Glu	ATA Ile	TTT Phe	GAA Glu	AAT Asn	AAA Lys	ATT Ile	ATT Ile	ACA Thr	GAT Asp	GAG Glu	1440
	465				470					475					480	
ACT Thr	AAT Asn	GTA Val	CAA Gln	AAT Asn	TAT Tyr	TCA Ser	GAT Asp	AAT Asn	TTT Phe	TCA Ser	TTA Leu	GAT Asp	GAA Glu	TCT Ser	ATT Ile	1488
				485					490					495		
TTA Leu	GAT Asp	GGG Gly	CAA Gln	GTT Val	CCT Pro	ATT Ile	AAT Asn	CCT Pro	GAA Glu	ATA Ile	GTA Val	GAT Asp	CCA Pro	CTA Leu	TTA Leu	1536
		500						505					510			
CCC Pro	AAT Asn	GTT Val	AAT Asn	ATG Met	GAA Glu	CCT Pro	TTA Leu	AAT Asn	CTT Leu	CCA Pro	GGT Gly	GAA Glu	GAA Glu	ATA Ile	GTA Val	1584
		515					520					525				
TTT Phe	TAT Tyr	GAT Asp	GAT Asp	ATT Ile	ACT Thr	AAA Lys	TAT Tyr	GTT Val	GAT Asp	TAT Tyr	TTA Leu	AAT Asn	TCT Ser	TAT Tyr	TAT Tyr	1632
	530					535					540					
TAT Tyr	TTG Leu	GAA Glu	TCT Ser	CAA Gln	AAA Lys	TTA Leu	AGT Ser	AAT Asn	AAT Asn	GTT Val	GAA Glu	AAT Asn	ATT Ile	ACT Thr	CTT Leu	1680
	545				550					555					560	
ACA Thr	ACT Thr	TCA Ser	GTT Val	GAA Glu	GAA Glu	GCA Ala	TTA Leu	GGT Gly	TAT Tyr	AGC Ser	AAT Asn	AAG Lys	ATA Ile	TAC Tyr	ACA Thr	1728
				565				570						575		
TTT Phe	TTA Leu	CCT Pro	AGC Ser	TTA Leu	GCT Ala	GAA Glu	AAA Lys	GTG Val	AAT Asn	AAA Lys	GGT Gly	GTT Val	CAA Gln	GCA Ala	GGT Gly	1776
		580						585					590			

TTA TTC TTA AAT TGG GCG AAT GAA GTA GTT GAG GAT TTT ACT ACA AAT Leu Phe Leu Asn Trp Ala Asn Glu Val Val Glu Asp Phe Thr Thr Asn 595 600 605	1824
ATT ATG AAG AAA GAT ACA TTG GAT AAA ATA TCA GAT GTA TCA GTA ATA Ile Met Lys Lys Asp Thr Leu Asp Lys Ile Ser Asp Val Ser Val Ile 610 615 620	1872
ATT CCA TAT ATA GGA CCT GCC TTA AAT ATA GGA AAT TCA GCA TTA AGG Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Gly Asn Ser Ala Leu Arg 625 630 635 640	1920
GGA AAT TTT AAG CAA GCA TTT GCA ACA GCT GGT GTA GCT TTT TTA TTA Gly Asn Phe Lys Gln Ala Phe Ala Thr Ala Gly Val Ala Phe Leu Leu 645 650 655	1968
GAG GGA TTT CCA GAG TTT ACT ATA CCT GCA CTC GGT GTA TTT ACC TTT Glu Gly Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly Val Phe Thr Phe 660 665 670	2016
TAT AGT TCT ATT CAA GAA AGA GAG AAA ATT ATT AAA ACT ATA GAA AAT Tyr Ser Ser Ile Gln Glu Arg Glu Lys Ile Ile Lys Thr Ile Glu Asn 675 680 685	2064
TGT TTG GAA CAA AGA GTT AAG AGA TGG AAA GAT TCA TAT CAA TGG ATG Cys Leu Glu Gln Arg Val Lys Arg Trp Lys Asp Ser Tyr Gln Trp Met 690 695 700	2112
GTA TCA AAT TGG TTG TCA AGA ATT ACT ACT CAA TTT AAT CAT ATA AAT Val Ser Asn Trp Leu Ser Arg Ile Thr Thr Gln Phe Asn His Ile Asn 705 710 715 720	2160
TAT CAA ATG TAT GAT TCT TTA AGT TAT CAG GCA GAT GCA ATC AAA GCT Tyr Gln Met Tyr Asp Ser Leu Ser Tyr Gln Ala Asp Ala Ile Lys Ala 725 730 735	2208
AAA ATA GAT TTA GAA TAT AAA AAA TAC TCA GGA AGT GAT AAA GAA AAT Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser Asp Lys Glu Asn 740 745 750	2256
ATA AAA AGT CAA GTT GAA AAT TTA AAA AAT AGT TTA GAT GTA AAA ATT Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu Asp Val Lys Ile 755 760 765	2304
TCG GAA GCA ATG AAT AAT ATA AAT AAA TTT ATA CGA GAA TGT TCT GTA Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg Glu Cys Ser Val 770 775 780	2352
ACA TAC TTA TTT AAA AAT ATG CTC CCT AAA GTA ATT GAC GAA TTA AAT Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile Asp Glu Leu Asn 785 790 795 800	2400
AAG TTT GAT TTA AGA ACT AAA ACA GAA TTA ATT AAT CTT ATA GAT AGT Lys Phe Asp Leu Arg Thr Lys Thr Glu Leu Ile Asn Leu Ile Asp Ser 805 810 815	2448
CAT AAT ATT ATT CTA GTT GGT GAA GTA GAT AGA TTA AAA GCA AAA GTA His Asn Ile Ile Leu Val Gly Glu Val Asp Arg Leu Lys Ala Lys Val 820 825 830	2496

AAT GAG AGT TTT GAA AAT ACA ATG CCT TTT AAT ATT TTT TCA TAT ACT Asn Glu Ser Phe Glu Asn Thr Met Pro Phe Asn Ile Phe Ser Tyr Thr 835 840 845	2544
AAT AAT TCT TTA TTA AAA GAT ATA ATT AAT GAA TAT TTC AAT AGT ATT Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe Asn Ser Ile 850 855 860	2592
AAT GAT TCA AAA ATT TTG AGC TTA CAA AAC AAA AAA AAT GCT TTA GTG Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Lys Lys Asn Ala Leu Val 865 870 875 880	2640
GAT ACA TCA GGA TAT AAT GCA GAA GTG AGG GTA GGA GAT AAT GTT CAA Asp Thr Ser Gly Tyr Asn Ala Glu Val Arg Val Gly Asp Asn Val Gln 885 890 895	2688
CTT AAT ACG ATA TAT ACA AAT GAC TTT AAA TTA AGT AGT TCA GGA GAT Leu Asn Thr Ile Tyr Thr Asn Asp Phe Lys Leu Ser Ser Ser Gly Asp 900 905 910	2736
AAA ATT ATA GTA AAT TTA AAT AAT AAT ATT TTA TAT AGC GCT ATT TAT Lys Ile Ile Val Asn Leu Asn Asn Asn Ile Leu Tyr Ser Ala Ile Tyr 915 920 925	2784
GAG AAC TCT AGT GTT AGT TTT TGG ATT AAG ATA TCT AAA GAT TTA ACT Glu Asn Ser Ser Val Ser Phe Trp Ile Lys Ile Ser Lys Asp Leu Thr 930 935 940	2832
AAT TCT CAT AAT GAA TAT ACA ATA ATT AAC AGT ATA GAA CAA AAT TCT Asn Ser His Asn Glu Tyr Thr Ile Ile Asn Ser Ile Glu Gln Asn Ser 945 950 955 960	2880
GGG TGG AAA TTA TGT ATT AGG AAT GGC AAT ATA GAA TGG ATT TTA CAA Gly Trp Lys Leu Cys Ile Arg Asn Gly Asn Ile Glu Trp Ile Leu Gln 965 970 975	2928
GAT GTT AAT AGA AAG TAT AAA AGT TTA ATT TTT GAT TAT AGT GAA TCA Asp Val Asn Arg Lys Tyr Lys Ser Leu Ile Phe Asp Tyr Ser Glu Ser 980 985 990	2976
TTA AGT CAT ACA GGA TAT ACA AAT AAA TGG TTT TTT GTT ACT ATA ACT Leu Ser His Thr Gly Tyr Thr Asn Lys Trp Phe Phe Val Thr Ile Thr 995 1000 1005	3024
AAT AAT ATA ATG GGG TAT ATG AAA CTT TAT ATA AAT GGA GAA TTA AAG Asn Asn Ile Met Gly Tyr Met Lys Leu Tyr Ile Asn Gly Glu Leu Lys 1010 1015 1020	3072
CAG AGT CAA AAA ATT GAA GAT TTA GAT GAG GTT AAG TTA GAT AAA ACC Gln Ser Gln Lys Ile Glu Asp Leu Asp Glu Val Lys Leu Asp Lys Thr 1025 1030 1035 1040	3120
ATA GTA TTT GGA ATA GAT GAG AAT ATA GAT GAG AAT CAG ATG CTT TGG Ile Val Phe Gly Ile Asp Glu Asn Ile Asp Glu Asn Gln Met Leu Trp 1045 1050 1055	3168
ATT AGA GAT TTT AAT ATT TTT TCT AAA GAA TTA AGT AAT GAA GAT ATT Ile Arg Asp Phe Asn Ile Phe Ser Lys Glu Leu Ser Asn Glu Asp Ile 1060 1065 1070	3216

AAT ATT GTA TAT GAG GGA CAA ATA TTA AGA AAT GTT ATT AAA GAT TAT Asn Ile Val Tyr Glu Gly Gln Ile Leu Arg Asn Val Ile Lys Asp Tyr 1075 1080 1085	3264
TGG GGA AAT CCT TTG AAG TTT GAT ACA GAA TAT TAT ATT ATT AAT GAT Trp Gly Asn Pro Leu Lys Phe Asp Thr Glu Tyr Tyr Ile Ile Asn Asp 1090 1095 1100	3312
AAT TAT ATA GAT AGG TAT ATT GCA CCT GAA AGT AAT GTA CTT GTA CTT Asn Tyr Ile Asp Arg Tyr Ile Ala Pro Glu Ser Asn Val Leu Val Leu 1105 1110 1115 1120	3360
GTT CGG TAT CCA GAT AGA TCT AAA TTA TAT ACT GGA AAT CCT ATT ACT Val Arg Tyr Pro Asp Arg Ser Lys Leu Tyr Thr Gly Asn Pro Ile Thr 1125 1130 1135	3408
ATT AAA TCA GTA TCT GAT AAG AAT CCT TAT AGT AGA ATT TTA AAT GGA Ile Lys Ser Val Ser Asp Lys Asn Pro Tyr Ser Arg Ile Leu Asn Gly 1140 1145 1150	3456
GAT AAT ATA ATT CTT CAT ATG TTA TAT AAT AGT AGG AAA TAT ATG ATA Asp Asn Ile Ile Leu His Met Leu Tyr Asn Ser Arg Lys Tyr Met Ile 1155 1160 1165	3504
ATA AGA GAT ACT GAT ACA ATA TAT GCA ACA CAA GGA GGA GAG TGT TCA Ile Arg Asp Thr Asp Thr Ile Tyr Ala Thr Gln Gly Gly Glu Cys Ser 1170 1175 1180	3552
CAA AAT TGT GTA TAT GCA TTA AAA TTA CAG AGT AAT TTA GGT AAT TAT Gln Asn Cys Val Tyr Ala Leu Lys Leu Gln Ser Asn Leu Gly Asn Tyr 1185 1190 1195 1200	3600
GGT ATA GGT ATA TTT AGT ATA AAA AAT ATT GTA TCT AAA AAT AAA TAT Gly Ile Gly Ile Phe Ser Ile Lys Asn Ile Val Ser Lys Asn Lys Tyr 1205 1210 1215	3648
TGT AGT CAA ATT TTC TCT AGT TTT AGG GAA AAT ACA ATG CTT CTA GCA Cys Ser Gln Ile Phe Ser Ser Phe Arg Glu Asn Thr Met Leu Leu Ala 1220 1225 1230	3696
GAT ATA TAT AAA CCT TGG AGA TTT TCT TTT AAA AAT GCA TAC ACG CCA Asp Ile Tyr Lys Pro Trp Arg Phe Ser Phe Lys Asn Ala Tyr Thr Pro 1235 1240 1245	3744
GTT GCA GTA ACT AAT TAT GAA ACA AAA CTA TTA TCA ACT TCA TCT TTT Val Ala Val Thr Asn Tyr Glu Thr Lys Leu Leu Ser Thr Ser Ser Phe 1250 1255 1260	3792
TGG AAA TTT ATT TCT AGG GAT CCA GGA TGG GTA GAG TAA Trp Lys Phe Ile Ser Arg Asp Pro Gly Trp Val Glu 1265 1270 1275	3831

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1276 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

Met Thr Trp Pro Val Lys Asp Phe Asn Tyr Ser Asp Pro Val Asn Asp  
1 5 10 15  
Asn Asp Ile Leu Tyr Leu Arg Ile Pro Gln Asn Lys Leu Ile Thr Thr  
20 25 30  
Pro Val Lys Ala Phe Met Ile Thr Gln Asn Ile Trp Val Ile Pro Glu  
35 40 45  
Arg Phe Ser Ser Asp Thr Asn Pro Ser Leu Ser Lys Pro Pro Arg Pro  
50 55 60  
Thr Ser Lys Tyr Gln Ser Tyr Tyr Asp Pro Ser Tyr Leu Ser Thr Asp  
65 70 75 80  
Glu Gln Lys Asp Thr Phe Leu Lys Gly Ile Ile Lys Leu Phe Lys Arg  
85 90 95  
Ile Asn Glu Arg Asp Ile Gly Lys Lys Leu Ile Asn Tyr Leu Val Val  
100 105 110  
Gly Ser Pro Phe Met Gly Asp Ser Ser Thr Pro Glu Asp Thr Phe Asp  
115 120 125  
Phe Thr Arg His Thr Thr Asn Ile Ala Val Glu Lys Phe Glu Asn Gly  
130 135 140  
Ser Trp Lys Val Thr Asn Ile Ile Thr Pro Ser Val Leu Ile Phe Gly  
145 150 155 160  
Pro Leu Pro Asn Ile Leu Asp Tyr Thr Ala Ser Leu Thr Leu Gln Gly  
165 170 175  
Gln Gln Ser Asn Pro Ser Phe Glu Gly Phe Gly Thr Leu Ser Ile Leu  
180 185 190  
Lys Val Ala Pro Glu Phe Leu Leu Thr Phe Ser Asp Val Thr Ser Asn  
195 200 205  
Gln Ser Ser Ala Val Leu Gly Lys Ser Ile Phe Cys Met Asp Pro Val  
210 215 220  
Ile Ala Leu Met His Glu Leu Thr His Ser Leu His Gln Leu Tyr Gly  
225 230 235 240  
Ile Asn Ile Pro Ser Asp Lys Arg Ile Arg Pro Gln Val Ser Glu Gly  
245 250 255  
Phe Phe Ser Gln Asp Gly Pro Asn Val Gln Phe Glu Glu Leu Tyr Thr  
260 265 270  
Phe Gly Gly Leu Asp Val Glu Ile Ile Pro Gln Ile Glu Arg Ser Gln  
275 280 285  
Leu Arg Glu Lys Ala Leu Gly His Tyr Lys Asp Ile Ala Lys Arg Leu  
290 295 300  
Asn Asn Ile Asn Lys Thr Ile Pro Ser Ser Trp Ile Ser Asn Ile Asp  
305 310 315 320

Lys Tyr Lys Lys Ile Phe Ser Glu Lys Tyr Asn Phe Asp Lys Asp Asn  
 325 330 335  
 Thr Gly Asn Phe Val Val Asn Ile Asp Lys Phe Asn Ser Leu Tyr Ser  
 340 345 350  
 Asp Leu Thr Asn Val Met Ser Glu Val Val Tyr Ser Ser Gln Tyr Asn  
 355 360 365  
 Val Lys Asn Arg Thr His Tyr Phe Ser Arg His Tyr Leu Pro Val Phe  
 370 375 380  
 Ala Asn Ile Leu Asp Asp Asn Ile Tyr Thr Ile Arg Asp Gly Phe Asn  
 385 390 395 400  
 Leu Thr Asn Lys Gly Phe Asn Ile Glu Asn Ser Gly Gln Asn Ile Glu  
 405 410 415  
 Arg Asn Pro Ala Leu Gln Lys Leu Ser Ser Glu Ser Val Val Asp Leu  
 420 425 430  
 Phe Thr Lys Val Cys Leu Arg Leu Thr Lys Asn Ser Arg Asp Asp Ser  
 435 440 445  
 Thr Cys Ile Lys Val Lys Asn Asn Arg Leu Pro Tyr Val Ala Asp Lys  
 450 455 460  
 Asp Ser Ile Ser Gln Glu Ile Phe Glu Asn Lys Ile Ile Thr Asp Glu  
 465 470 475 480  
 Thr Asn Val Gln Asn Tyr Ser Asp Asn Phe Ser Leu Asp Glu Ser Ile  
 485 490 495  
 Leu Asp Gly Gln Val Pro Ile Asn Pro Glu Ile Val Asp Pro Leu Leu  
 500 505 510  
 Pro Asn Val Asn Met Glu Pro Leu Asn Leu Pro Gly Glu Glu Ile Val  
 515 520 525  
 Phe Tyr Asp Asp Ile Thr Lys Tyr Val Asp Tyr Leu Asn Ser Tyr Tyr  
 530 535 540  
 Tyr Leu Glu Ser Gln Lys Leu Ser Asn Asn Val Glu Asn Ile Thr Leu  
 545 550 555 560  
 Thr Thr Ser Val Glu Glu Ala Leu Gly Tyr Ser Asn Lys Ile Tyr Thr  
 565 570 575  
 Phe Leu Pro Ser Leu Ala Glu Lys Val Asn Lys Gly Val Gln Ala Gly  
 580 585 590  
 Leu Phe Leu Asn Trp Ala Asn Glu Val Val Glu Asp Phe Thr Thr Asn  
 595 600 605  
 Ile Met Lys Lys Asp Thr Leu Asp Lys Ile Ser Asp Val Ser Val Ile  
 610 615 620  
 Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Gly Asn Ser Ala Leu Arg  
 625 630 635 640  
 Gly Asn Phe Lys Gln Ala Phe Ala Thr Ala Gly Val Ala Phe Leu Leu  
 645 650 655



Glu Gly Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly Val Phe Thr Phe  
 660 665 670  
 Tyr Ser Ser Ile Gln Glu Arg Glu Lys Ile Ile Lys Thr Ile Glu Asn  
 675 680 685  
 Cys Leu Glu Gln Arg Val Lys Arg Trp Lys Asp Ser Tyr Gln Trp Met  
 690 695 700  
 Val Ser Asn Trp Leu Ser Arg Ile Thr Thr Gln Phe Asn His Ile Asn  
 705 710 715 720  
 Tyr Gln Met Tyr Asp Ser Leu Ser Tyr Gln Ala Asp Ala Ile Lys Ala  
 725 730 735  
 Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser Asp Lys Glu Asn  
 740 745 750  
 Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu Asp Val Lys Ile  
 755 760 765  
 Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg Glu Cys Ser Val  
 770 775 780  
 Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile Asp Glu Leu Asn  
 785 790 795 800  
 Lys Phe Asp Leu Arg Thr Lys Thr Glu Leu Ile Asn Leu Ile Asp Ser  
 805 810 815  
 His Asn Ile Ile Leu Val Gly Glu Val Asp Arg Leu Lys Ala Lys Val  
 820 825 830  
 Asn Glu Ser Phe Glu Asn Thr Met Pro Phe Asn Ile Phe Ser Tyr Thr  
 835 840 845  
 Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe Asn Ser Ile  
 850 855 860  
 Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Lys Lys Asn Ala Leu Val  
 865 870 875 880  
 Asp Thr Ser Gly Tyr Asn Ala Glu Val Arg Val Gly Asp Asn Val Gln  
 885 890 895  
 Leu Asn Thr Ile Tyr Thr Asn Asp Phe Lys Leu Ser Ser Ser Gly Asp  
 900 905 910  
 Lys Ile Ile Val Asn Leu Asn Asn Asn Ile Leu Tyr Ser Ala Ile Tyr  
 915 920 925  
 Glu Asn Ser Ser Val Ser Phe Trp Ile Lys Ile Ser Lys Asp Leu Thr  
 930 935 940  
 Asn Ser His Asn Glu Tyr Thr Ile Ile Asn Ser Ile Glu Gln Asn Ser  
 945 950 955 960  
 Gly Trp Lys Leu Cys Ile Arg Asn Gly Asn Ile Glu Trp Ile Leu Gln  
 965 970 975  
 Asp Val Asn Arg Lys Tyr Lys Ser Leu Ile Phe Asp Tyr Ser Glu Ser  
 980 985 990

Leu Ser His Thr Gly Tyr Thr Asn Lys Trp Phe Phe Val Thr Ile Thr  
 995 1000 1005  
 Asn Asn Ile Met Gly Tyr Met Lys Leu Tyr Ile Asn Gly Glu Leu Lys  
 1010 1015 1020  
 Gln Ser Gln Lys Ile Glu Asp Leu Asp Glu Val Lys Leu Asp Lys Thr  
 1025 1030 1035 1040  
 Ile Val Phe Gly Ile Asp Glu Asn Ile Asp Glu Asn Gln Met Leu Trp  
 1045 1050 1055  
 Ile Arg Asp Phe Asn Ile Phe Ser Lys Glu Leu Ser Asn Glu Asp Ile  
 1060 1065 1070  
 Asn Ile Val Tyr Glu Gly Gln Ile Leu Arg Asn Val Ile Lys Asp Tyr  
 1075 1080 1085  
 Trp Gly Asn Pro Leu Lys Phe Asp Thr Glu Tyr Tyr Ile Ile Asn Asp  
 1090 1095 1100  
 Asn Tyr Ile Asp Arg Tyr Ile Ala Pro Glu Ser Asn Val Leu Val Leu  
 1105 1110 1115 1120  
 Val Arg Tyr Pro Asp Arg Ser Lys Leu Tyr Thr Gly Asn Pro Ile Thr  
 1125 1130 1135  
 Ile Lys Ser Val Ser Asp Lys Asn Pro Tyr Ser Arg Ile Leu Asn Gly  
 1140 1145 1150  
 Asp Asn Ile Ile Leu His Met Leu Tyr Asn Ser Arg Lys Tyr Met Ile  
 1155 1160 1165  
 Ile Arg Asp Thr Asp Thr Ile Tyr Ala Thr Gln Gly Gly Glu Cys Ser  
 1170 1175 1180  
 Gln Asn Cys Val Tyr Ala Leu Lys Leu Gln Ser Asn Leu Gly Asn Tyr  
 1185 1190 1195 1200  
 Gly Ile Gly Ile Phe Ser Ile Lys Asn Ile Val Ser Lys Asn Lys Tyr  
 1205 1210 1215  
 Cys Ser Gln Ile Phe Ser Ser Phe Arg Glu Asn Thr Met Leu Leu Ala  
 1220 1225 1230  
 Asp Ile Tyr Lys Pro Trp Arg Phe Ser Phe Lys Asn Ala Tyr Thr Pro  
 1235 1240 1245  
 Val Ala Val Thr Asn Tyr Glu Thr Lys Leu Leu Ser Thr Ser Ser Phe  
 1250 1255 1260  
 Trp Lys Phe Ile Ser Arg Asp Pro Gly Trp Val Glu  
 1265 1270 1275

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1469 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 108..1460

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

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AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA      60
TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACC ATG GGC CAT      116
                                         Met Gly His
                                         1

CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT      164
His His His His His His His His His Ser Ser Gly His Ile Glu Gly
      5                      10                      15

CGT CAT ATG GCT AGC ATG GCT TTA TTA AAA GAT ATA ATT AAT GAA TAT      212
Arg His Met Ala Ser Met Ala Leu Leu Lys Asp Ile Ile Asn Glu Tyr
      20                      25                      30                      35

TTC AAT AGT ATT AAT GAT TCA AAA ATT TTG AGC TTA CAA AAC AAA AAA      260
Phe Asn Ser Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Lys Lys
                      40                      45                      50

AAT GCT TTA GTG GAT ACA TCA GGA TAT AAT GCA GAA GTG AGG GTA GGA      308
Asn Ala Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Arg Val Gly
                      55                      60                      65

GAT AAT GTT CAA CTT AAT ACG ATA TAT ACA AAT GAC TTT AAA TTA AGT      356
Asp Asn Val Gln Leu Asn Thr Ile Tyr Thr Asn Asp Phe Lys Leu Ser
                      70                      75                      80

AGT TCA GGA GAT AAA ATT ATA GTA AAT TTA AAT AAT AAT ATT TTA TAT      404
Ser Ser Gly Asp Lys Ile Ile Val Asn Leu Asn Asn Asn Ile Leu Tyr
                      85                      90                      95

AGC GCT ATT TAT GAG AAC TCT AGT GTT AGT TTT TGG ATT AAG ATA TCT      452
Ser Ala Ile Tyr Glu Asn Ser Ser Val Ser Phe Trp Ile Lys Ile Ser
      100                      105                      110                      115

AAA GAT TTA ACT AAT TCT CAT AAT GAA TAT ACA ATA ATT AAC AGT ATA      500
Lys Asp Leu Thr Asn Ser His Asn Glu Tyr Thr Ile Ile Asn Ser Ile
                      120                      125                      130

GAA CAA AAT TCT GGG TGG AAA TTA TGT ATT AGG AAT GGC AAT ATA GAA      548
Glu Gln Asn Ser Gly Trp Lys Leu Cys Ile Arg Asn Gly Asn Ile Glu
                      135                      140                      145

TGG ATT TTA CAA GAT GTT AAT AGA AAG TAT AAA AGT TTA ATT TTT GAT      596
Trp Ile Leu Gln Asp Val Asn Arg Lys Tyr Lys Ser Leu Ile Phe Asp
                      150                      155                      160
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TAT AGT GAA TCA TTA AGT CAT ACA GGA TAT ACA AAT AAA TGG TTT TTT Tyr Ser Glu Ser Leu Ser His Thr Gly Tyr Thr Asn Lys Trp Phe Phe 165 170 175	644
GTT ACT ATA ACT AAT AAT ATA ATG GGG TAT ATG AAA CTT TAT ATA AAT Val Thr Ile Thr Asn Asn Ile Met Gly Tyr Met Lys Leu Tyr Ile Asn 180 185 190 195	692
GGA GAA TTA AAG CAG AGT CAA AAA ATT GAA GAT TTA GAT GAG GTT AAG Gly Glu Leu Lys Gln Ser Gln Lys Ile Glu Asp Leu Asp Glu Val Lys 200 205 210	740
TTA GAT AAA ACC ATA GTA TTT GGA ATA GAT GAG AAT ATA GAT GAG AAT Leu Asp Lys Thr Ile Val Phe Gly Ile Asp Glu Asn Ile Asp Glu Asn 215 220 225	788
CAG ATG CTT TGG ATT AGA GAT TTT AAT ATT TTT TCT AAA GAA TTA AGT Gln Met Leu Trp Ile Arg Asp Phe Asn Ile Phe Ser Lys Glu Leu Ser 230 235 240	836
AAT GAA GAT ATT AAT ATT GTA TAT GAG GGA CAA ATA TTA AGA AAT GTT Asn Glu Asp Ile Asn Ile Val Tyr Glu Gly Gln Ile Leu Arg Asn Val 245 250 255	884
ATT AAA GAT TAT TGG GGA AAT CCT TTG AAG TTT GAT ACA GAA TAT TAT Ile Lys Asp Tyr Trp Gly Asn Pro Leu Lys Phe Asp Thr Glu Tyr Tyr 260 265 270 275	932
ATT ATT AAT GAT AAT TAT ATA GAT AGG TAT ATT GCA CCT GAA AGT AAT Ile Ile Asn Asp Asn Tyr Ile Asp Arg Tyr Ile Ala Pro Glu Ser Asn 280 285 290	980
GTA CTT GTA CTT GTT CGG TAT CCA GAT AGA TCT AAA TTA TAT ACT GGA Val Leu Val Leu Val Arg Tyr Pro Asp Arg Ser Lys Leu Tyr Thr Gly 295 300 305	1028
AAT CCT ATT ACT ATT AAA TCA GTA TCT GAT AAG AAT CCT TAT AGT AGA Asn Pro Ile Thr Ile Lys Ser Val Ser Asp Lys Asn Pro Tyr Ser Arg 310 315 320	1076
ATT TTA AAT GGA GAT AAT ATA ATT CTT CAT ATG TTA TAT AAT AGT AGG Ile Leu Asn Gly Asp Asn Ile Ile Leu His Met Leu Tyr Asn Ser Arg 325 330 335	1124
AAA TAT ATG ATA ATA AGA GAT ACT GAT ACA ATA TAT GCA ACA CAA GGA Lys Tyr Met Ile Ile Arg Asp Thr Asp Thr Ile Tyr Ala Thr Gln Gly 340 345 350 355	1172
GGA GAG TGT TCA CAA AAT TGT GTA TAT GCA TTA AAA TTA CAG AGT AAT Gly Glu Cys Ser Gln Asn Cys Val Tyr Ala Leu Lys Leu Gln Ser Asn 360 365 370	1220
TTA GGT AAT TAT GGT ATA GGT ATA TTT AGT ATA AAA AAT ATT GTA TCT Leu Gly Asn Tyr Gly Ile Gly Ile Phe Ser Ile Lys Asn Ile Val Ser 375 380 385	1268
AAA AAT AAA TAT TGT AGT CAA ATT TTC TCT AGT TTT AGG GAA AAT ACA Lys Asn Lys Tyr Cys Ser Gln Ile Phe Ser Ser Phe Arg Glu Asn Thr 390 395 400	1316

ATG CTT CTA GCA GAT ATA TAT AAA CCT TGG AGA TTT TCT TTT AAA AAT	1364
Met Leu Leu Ala Asp Ile Tyr Lys Pro Trp Arg Phe Ser Phe Lys Asn	
405 410 415	

GCA TAC ACG CCA GTT GCA GTA ACT AAT TAT GAA ACA AAA CTA TTA TCA	1412
Ala Tyr Thr Pro Val Ala Val Thr Asn Tyr Glu Thr Lys Leu Leu Ser	
420 425 430 435	

ACT TCA TCT TTT TGG AAA TTT ATT TCT AGG GAT CCA GGA TGG GTA GAG	1460
Thr Ser Ser Phe Trp Lys Phe Ile Ser Arg Asp Pro Gly Trp Val Glu	
440 445 450	

TAAAAGCTT	1469
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(2) INFORMATION FOR SEQ ID NO:68:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 451 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Met Gly His His His His His His His His His His His Ser Ser Gly His	
1 5 10 15	
Ile Glu Gly Arg His Met Ala Ser Met Ala Leu Leu Lys Asp Ile Ile	
20 25 30	
Asn Glu Tyr Phe Asn Ser Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln	
35 40 45	
Asn Lys Lys Asn Ala Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val	
50 55 60	
Arg Val Gly Asp Asn Val Gln Leu Asn Thr Ile Tyr Thr Asn Asp Phe	
65 70 75 80	
Lys Leu Ser Ser Ser Gly Asp Lys Ile Ile Val Asn Leu Asn Asn Asn	
85 90 95	
Ile Leu Tyr Ser Ala Ile Tyr Glu Asn Ser Ser Val Ser Phe Trp Ile	
100 105 110	
Lys Ile Ser Lys Asp Leu Thr Asn Ser His Asn Glu Tyr Thr Ile Ile	
115 120 125	
Asn Ser Ile Glu Gln Asn Ser Gly Trp Lys Leu Cys Ile Arg Asn Gly	
130 135 140	
Asn Ile Glu Trp Ile Leu Gln Asp Val Asn Arg Lys Tyr Lys Ser Leu	
145 150 155 160	
Ile Phe Asp Tyr Ser Glu Ser Leu Ser His Thr Gly Tyr Thr Asn Lys	
165 170 175	
Trp Phe Phe Val Thr Ile Thr Asn Asn Ile Met Gly Tyr Met Lys Leu	
180 185 190	

Tyr Ile Asn Gly Glu Leu Lys Gln Ser Gln Lys Ile Glu Asp Leu Asp  
 195 200 205  
 Glu Val Lys Leu Asp Lys Thr Ile Val Phe Gly Ile Asp Glu Asn Ile  
 210 215 220  
 Asp Glu Asn Gln Met Leu Trp Ile Arg Asp Phe Asn Ile Phe Ser Lys  
 225 230 235 240  
 Glu Leu Ser Asn Glu Asp Ile Asn Ile Val Tyr Glu Gly Gln Ile Leu  
 245 250 255  
 Arg Asn Val Ile Lys Asp Tyr Trp Gly Asn Pro Leu Lys Phe Asp Thr  
 260 265 270  
 Glu Tyr Tyr Ile Ile Asn Asp Asn Tyr Ile Asp Arg Tyr Ile Ala Pro  
 275 280 285  
 Glu Ser Asn Val Leu Val Leu Val Arg Tyr Pro Asp Arg Ser Lys Leu  
 290 295 300  
 Tyr Thr Gly Asn Pro Ile Thr Ile Lys Ser Val Ser Asp Lys Asn Pro  
 305 310 315 320  
 Tyr Ser Arg Ile Leu Asn Gly Asp Asn Ile Ile Leu His Met Leu Tyr  
 325 330 335  
 Asn Ser Arg Lys Tyr Met Ile Ile Arg Asp Thr Asp Thr Ile Tyr Ala  
 340 345 350  
 Thr Gln Gly Gly Glu Cys Ser Gln Asn Cys Val Tyr Ala Leu Lys Leu  
 355 360 365  
 Gln Ser Asn Leu Gly Asn Tyr Gly Ile Gly Ile Phe Ser Ile Lys Asn  
 370 375 380  
 Ile Val Ser Lys Asn Lys Tyr Cys Ser Gln Ile Phe Ser Ser Phe Arg  
 385 390 395 400  
 Glu Asn Thr Met Leu Leu Ala Asp Ile Tyr Lys Pro Trp Arg Phe Ser  
 405 410 415  
 Phe Lys Asn Ala Tyr Thr Pro Val Ala Val Thr Asn Tyr Glu Thr Lys  
 420 425 430  
 Leu Leu Ser Thr Ser Ser Phe Trp Lys Phe Ile Ser Arg Asp Pro Gly  
 435 440 445  
 Trp Val Glu  
 450

(2) INFORMATION FOR SEQ ID NO:69:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

GCAAGCTTTT ACTCTACCCA TCCTGGATCC CT

32

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3825 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..3822

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

ATG CCA GTT GCA ATA AAT AGT TTT AAT TAT AAT GAC CCT GTT AAT GAT	48
Met Pro Val Ala Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp	
1 5 10 15	
GAT ACA ATT TTA TAC ATG CAG ATA CCA TAT GAA GAA AAA AGT AAA AAA	96
Asp Thr Ile Leu Tyr Met Gln Ile Pro Tyr Glu Glu Lys Ser Lys Lys	
20 25 30	
TAT TAT AAA GCT TTT GAG ATT ATG CGT AAT GTT TGG ATA ATT CCT GAG	144
Tyr Tyr Lys Ala Phe Glu Ile Met Arg Asn Val Trp Ile Ile Pro Glu	
35 40 45	
AGA AAT ACA ATA GGA ACG AAT CCT AGT GAT TTT GAT CCA CCG GCT TCA	192
Arg Asn Thr Ile Gly Thr Asn Pro Ser Asp Phe Asp Pro Pro Ala Ser	
50 55 60	
TTA AAG AAC GGA AGC AGT GCT TAT TAT GAT CCT AAT TAT TTA ACC ACT	240
Leu Lys Asn Gly Ser Ser Ala Tyr Tyr Asp Pro Asn Tyr Leu Thr Thr	
65 70 75 80	
GAT GCT GAA AAA GAT AGA TAT TTA AAA ACA ACG ATA AAA TTA TTT AAG	288
Asp Ala Glu Lys Asp Arg Tyr Leu Lys Thr Thr Ile Lys Leu Phe Lys	
85 90 95	
AGA ATT AAT AGT AAT CCT GCA GGG AAA GTT TTG TTA CAA GAA ATA TCA	336
Arg Ile Asn Ser Asn Pro Ala Gly Lys Val Leu Leu Gln Glu Ile Ser	
100 105 110	
TAT GCT AAA CCA TAT TTA GGA AAT GAC CAC ACG CCA ATT GAT GAA TTC	384
Tyr Ala Lys Pro Tyr Leu Gly Asn Asp His Thr Pro Ile Asp Glu Phe	
115 120 125	
TCT CCA GTT ACT AGA ACT ACA AGT GTT AAT ATA AAA TTA TCA ACT AAT	432
Ser Pro Val Thr Arg Thr Thr Ser Val Asn Ile Lys Leu Ser Thr Asn	
130 135 140	
GTT GAA AGT TCA ATG TTA TTG AAT CTT CTT GTA TTG GGA GCA GGA CCT	480
Val Glu Ser Ser Met Leu Leu Asn Leu Leu Val Leu Gly Ala Gly Pro	
145 150 155 160	

GAT ATA TTT GAA AGT TGT TGT TAC CCC GTT AGA AAA CTA ATA GAT CCA Asp Ile Phe Glu Ser Cys Cys Tyr Pro Val Arg Lys Leu Ile Asp Pro 165 170 175	528
GAT GTA GTT TAT GAT CCA AGT AAT TAT GGT TTT GGA TCA ATT AAT ATC Asp Val Val Tyr Asp Pro Ser Asn Tyr Gly Phe Gly Ser Ile Asn Ile 180 185 190	576
GTG ACA TTT TCA CCT GAG TAT GAA TAT ACT TTT AAT GAT ATT AGT GGA Val Thr Phe Ser Pro Glu Tyr Glu Tyr Thr Phe Asn Asp Ile Ser Gly 195 200 205	624
GGG CAT AAT AGT AGT ACA GAA TCA TTT ATT GCA GAT CCT GCA ATT TCA Gly His Asn Ser Ser Thr Glu Ser Phe Ile Ala Asp Pro Ala Ile Ser 210 215 220	672
CTA GCT CAT GAA TTG ATA CAT GCA CTG CAT GGA TTA TAC GGG GCT AGG Leu Ala His Glu Leu Ile His Ala Leu His Gly Leu Tyr Gly Ala Arg 225 230 235 240	720
GGA GTT ACT TAT GAA GAG ACT ATA GAA GTA AAG CAA GCA CCT CTT ATG Gly Val Thr Tyr Glu Glu Thr Ile Glu Val Lys Gln Ala Pro Leu Met 245 250 255	768
ATA GCC GAA AAA CCC ATA AGG CTA GAA GAA TTT TTA ACC TTT GGA GGT Ile Ala Glu Lys Pro Ile Arg Leu Glu Glu Phe Leu Thr Phe Gly Gly 260 265 270	816
CAG GAT TTA AAT ATT ATT ACT AGT GCT ATG AAG GAA AAA ATA TAT AAC Gln Asp Leu Asn Ile Ile Thr Ser Ala Met Lys Glu Lys Ile Tyr Asn 275 280 285	864
AAT CTT TTA GCT AAC TAT GAA AAA ATA GCT ACT AGA CTT AGT GAA GTT Asn Leu Leu Ala Asn Tyr Glu Lys Ile Ala Thr Arg Leu Ser Glu Val 290 295 300	912
AAT AGT GCT CCT CCT GAA TAT GAT ATT AAT GAA TAT AAA GAT TAT TTT Asn Ser Ala Pro Pro Glu Tyr Asp Ile Asn Glu Tyr Lys Asp Tyr Phe 305 310 315 320	960
CAA TGG AAG TAT GGG CTA GAT AAA AAT GCT GAT GGA AGT TAT ACT GTA Gln Trp Lys Tyr Gly Leu Asp Lys Asn Ala Asp Gly Ser Tyr Thr Val 325 330 335	1008
AAT GAA AAT AAA TTT AAT GAA ATT TAT AAA AAA TTA TAT AGT TTT ACA Asn Glu Asn Lys Phe Asn Glu Ile Tyr Lys Lys Leu Tyr Ser Phe Thr 340 345 350	1056
GAG AGT GAC TTA GCA AAT AAA TTT AAA GTA AAA TGT AGA AAT ACT TAT Glu Ser Asp Leu Ala Asn Lys Phe Lys Val Lys Cys Arg Asn Thr Tyr 355 360 365	1104
TTT ATT AAA TAT GAA TTT TTA AAA GTT CCA AAT TTG TTA GAT GAT GAT Phe Ile Lys Tyr Glu Phe Leu Lys Val Pro Asn Leu Leu Asp Asp Asp 370 375 380	1152
ATT TAT ACT GTA TCA GAG GGG TTT AAT ATA GGT AAT TTA GCA GTA AAC Ile Tyr Thr Val Ser Glu Gly Phe Asn Ile Gly Asn Leu Ala Val Asn 385 390 395 400	1200



AAT CGC GGA CAA AGT ATA AAG TTA AAT CCT AAA ATT ATT GAT TCC ATT Asn Arg Gly Gln Ser Ile Lys Leu Asn Pro Lys Ile Ile Asp Ser Ile 405 410 415	1248
CCA GAT AAA GGT CTA GTA GAA AAG ATC GTT AAA TTT TGT AAG AGC GTT Pro Asp Lys Gly Leu Val Glu Lys Ile Val Lys Phe Cys Lys Ser Val 420 425 430	1296
ATT CCT AGA AAA GGT ACA AAG GCG CCA CCG CGA CTA TGC ATT AGA GTA Ile Pro Arg Lys Gly Thr Lys Ala Pro Pro Arg Leu Cys Ile Arg Val 435 440 445	1344
AAT AAT AGT GAG TTA TTT TTT GTA GCT TCA GAA AGT AGC TAT AAT GAA Asn Asn Ser Glu Leu Phe Phe Val Ala Ser Glu Ser Ser Tyr Asn Glu 450 455 460	1392
AAT GAT ATT AAT ACA CCT AAA GAA ATT GAC GAT ACA ACA AAT CTA AAT Asn Asp Ile Asn Thr Pro Lys Glu Ile Asp Asp Thr Thr Asn Leu Asn 465 470 475 480	1440
AAT AAT TAT AGA AAT AAT TTA GAT GAA GTT ATT TTA GAT TAT AAT AGT Asn Asn Tyr Arg Asn Asn Leu Asp Glu Val Ile Leu Asp Tyr Asn Ser 485 490 495	1488
CAG ACA ATA CCT CAA ATA TCA AAT CGA ACA TTA AAT ACA CTT GTA CAA Gln Thr Ile Pro Gln Ile Ser Asn Arg Thr Leu Asn Thr Leu Val Gln 500 505 510	1536
GAC AAT AGT TAT GTG CCA AGA TAT GAT TCT AAT GGA ACA AGT GAA ATA Asp Asn Ser Tyr Val Pro Arg Tyr Asp Ser Asn Gly Thr Ser Glu Ile 515 520 525	1584
GAG GAA TAT GAT GTT GTT GAC TTT AAT GTA TTT TTC TAT TTA CAT GCA Glu Glu Tyr Asp Val Val Asp Phe Asn Val Phe Phe Tyr Leu His Ala 530 535 540	1632
CAA AAA GTG CCA GAA GGT GAA ACC AAT ATA AGT TTA ACT TCT TCA ATT Gln Lys Val Pro Glu Gly Glu Thr Asn Ile Ser Leu Thr Ser Ser Ile 545 550 555 560	1680
GAT ACA GCA TTA TTA GAA GAA TCC AAA GAT ATA TTT TTT TCT TCA GAG Asp Thr Ala Leu Leu Glu Glu Ser Lys Asp Ile Phe Phe Ser Ser Glu 565 570 575	1728
TTT ATC GAT ACT ATC AAT AAA CCT GTA AAT GCA GCA CTA TTT ATA GAT Phe Ile Asp Thr Ile Asn Lys Pro Val Asn Ala Ala Leu Phe Ile Asp 580 585 590	1776
TGG ATA AGC AAA GTA ATA AGA GAT TTT ACC ACT GAA GCT ACA CAA AAA Trp Ile Ser Lys Val Ile Arg Asp Phe Thr Thr Glu Ala Thr Gln Lys 595 600 605	1824
AGT ACT GTT GAT AAG ATT GCA GAC ATA TCT TTA ATT GTA CCC TAT GTA Ser Thr Val Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Val 610 615 620	1872
GGT CTT GCT TTG AAT ATA ATT ATT GAG GCA GAA AAA GGA AAT TTT GAG Gly Leu Ala Leu Asn Ile Ile Ile Glu Ala Glu Lys Gly Asn Phe Glu 625 630 635 640	1920

GAG GCA TTT GAA TTA TTA GGA GTG GGT ATT TTA TTA GAA TTT GTG CCA Glu Ala Phe Glu Leu Leu Gly Val Gly Ile Leu Leu Glu Phe Val Pro 645 650 655	1968
GAA CTT ACA ATT CCT GTA ATT TTA GTG TTT ACG ATA AAA TCC TAT ATA Glu Leu Thr Ile Pro Val Ile Leu Val Phe Thr Ile Lys Ser Tyr Ile 660 665 670	2016
GAT TCA TAT GAG AAT AAA AAT AAA GCA ATT AAA GCA ATA AAT AAT TCA Asp Ser Tyr Glu Asn Lys Asn Lys Ala Ile Lys Ala Ile Asn Asn Ser 675 680 685	2064
TTA ATC GAA AGA GAA GCA AAG TGG AAA GAA ATA TAT AGT TGG ATA GTA Leu Ile Glu Arg Glu Ala Lys Trp Lys Glu Ile Tyr Ser Trp Ile Val 690 695 700	2112
TCA AAT TGG CTT ACT AGA ATT AAT ACT CAA TTT AAT AAA AGA AAA GAG Ser Asn Trp Leu Thr Arg Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu 705 710 715 720	2160
CAA ATG TAT CAG GCT TTA CAA AAT CAA GTA GAT GCA ATA AAA ACA GCA Gln Met Tyr Gln Ala Leu Gln Asn Gln Val Asp Ala Ile Lys Thr Ala 725 730 735	2208
ATA GAA TAT AAA TAT AAT AAT TAT ACT TCA GAT GAG AAA AAT AGA CTT Ile Glu Tyr Lys Tyr Asn Asn Tyr Thr Ser Asp Glu Lys Asn Arg Leu 740 745 750	2256
GAA TCT GAA TAT AAT ATC AAT AAT ATA GAA GAA GAA TTG AAT AAA AAA Glu Ser Glu Tyr Asn Ile Asn Asn Ile Glu Glu Glu Leu Asn Lys Lys 755 760 765	2304
GTT TCT TTA GCA ATG AAA AAT ATA GAA AGA TTT ATG ACA GAA AGT TCT Val Ser Leu Ala Met Lys Asn Ile Glu Arg Phe Met Thr Glu Ser Ser 770 775 780	2352
ATA TCT TAT TTA ATG AAA TTA ATA AAT GAA GCC AAA GTT GGT AAA TTA Ile Ser Tyr Leu Met Lys Leu Ile Asn Glu Ala Lys Val Gly Lys Leu 785 790 795 800	2400
AAA AAA TAT GAT AAC CAT GTT AAG AGC GAT TTA TTA AAC TAT ATT CTC Lys Lys Tyr Asp Asn His Val Lys Ser Asp Leu Leu Asn Tyr Ile Leu 805 810 815	2448
GAC CAT AGA TCA ATC TTA GGA GAG CAG ACA AAT GAA TTA AGT GAT TTG Asp His Arg Ser Ile Leu Gly Glu Gln Thr Asn Glu Leu Ser Asp Leu 820 825 830	2496
GTG ACT AGT ACT TTG AAT AGT AGT ATT CCA TTT GAA CTT TCT TCA TAT Val Thr Ser Thr Leu Asn Ser Ser Ile Pro Phe Glu Leu Ser Ser Tyr 835 840 845	2544
ACT AAT GAT AAA ATT CTA ATT ATA TAT TTT AAT AGA TTA TAT AAA AAA Thr Asn Asp Lys Ile Leu Ile Ile Tyr Phe Asn Arg Leu Tyr Lys Lys 850 855 860	2592
ATT AAA GAT AGT TCT ATT TTA GAT ATG CGA TAT GAA AAT AAT AAA TTT Ile Lys Asp Ser Ser Ile Leu Asp Met Arg Tyr Glu Asn Asn Lys Phe 865 870 875 880	2640

ATA GAT ATC TCT GGA TAT GGT TCA AAT ATA AGC ATT AAT GGA AAC GTA Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile Ser Ile Asn Gly Asn Val 885 890 895	2688
TAT ATT TAT TCA ACA AAT AGA AAT CAA TTT GGA ATA TAT AAT AGT AGG Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe Gly Ile Tyr Asn Ser Arg 900 905 910	2736
CTT AGT GAA GTT AAT ATA GCT CAA AAT AAT GAT ATT ATA TAC AAT AGT Leu Ser Glu Val Asn Ile Ala Gln Asn Asn Asp Ile Ile Tyr Asn Ser 915 920 925	2784
AGA TAT CAA AAT TTT AGT ATT AGT TTC TGG GTA AGG ATT CCT AAA CAC Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Lys His 930 935 940	2832
TAC AAA CCT ATG AAT CAT AAT CGG GAA TAC ACT ATA ATA AAT TGT ATG Tyr Lys Pro Met Asn His Asn Arg Glu Tyr Thr Ile Ile Asn Cys Met 945 950 955 960	2880
GGG AAT AAT AAT TCG GGA TGG AAA ATA TCA CTT AGA ACT GTT AGA GAT Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser Leu Arg Thr Val Arg Asp 965 970 975	2928
TGT GAA ATA ATT TGG ACT TTA CAA GAT ACT TCT GGA AAT AAG GAA AAT Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr Ser Gly Asn Lys Glu Asn 980 985 990	2976
TTA ATT TTT AGG TAT GAA GAA CTT AAT AGG ATA TCT AAT TAT ATA AAT Leu Ile Phe Arg Tyr Glu Glu Leu Asn Arg Ile Ser Asn Tyr Ile Asn 995 1000 1005	3024
AAA TGG ATT TTT GTA ACT ATT ACT AAT AAT AGA TTA GGC AAT TCT AGA Lys Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Gly Asn Ser Arg 1010 1015 1020	3072
ATT TAC ATC AAT GGA AAT TTA ATA GTT GAA AAA TCA ATT TCG AAT TTA Ile Tyr Ile Asn Gly Asn Leu Ile Val Glu Lys Ser Ile Ser Asn Leu 1025 1030 1035 1040	3120
GGT GAT ATT CAT GTT AGT GAT AAT ATA TTA TTT AAA ATT GTT GGT TGT Gly Asp Ile His Val Ser Asp Asn Ile Leu Phe Lys Ile Val Gly Cys 1045 1050 1055	3168
GAT GAT GAA ACG TAT GTT GGT ATA AGA TAT TTT AAA GTT TTT AAT ACG Asp Asp Glu Thr Tyr Val Gly Ile Arg Tyr Phe Lys Val Phe Asn Thr 1060 1065 1070	3216
GAA TTA GAT AAA ACA GAA ATT GAG ACT TTA TAT AGT AAT GAG CCA GAT Glu Leu Asp Lys Thr Glu Ile Glu Thr Leu Tyr Ser Asn Glu Pro Asp 1075 1080 1085	3264
CCA AGT ATC TTA AAA AAC TAT TGG GGA AAT TAT TTG CTA TAT AAT AAA Pro Ser Ile Leu Lys Asn Tyr Trp Gly Asn Tyr Leu Leu Tyr Asn Lys 1090 1095 1100	3312
AAA TAT TAT TTA TTC AAT TTA CTA AGA AAA GAT AAG TAT ATT ACT CTG Lys Tyr Tyr Leu Phe Asn Leu Leu Arg Lys Asp Lys Tyr Ile Thr Leu 1105 1110 1115 1120	3360

AAT TCA GGC ATT TTA AAT ATT AAT CAA CAA AGA GGT GTT ACT GAA GGC Asn Ser Gly Ile Leu Asn Ile Asn Gln Gln Arg Gly Val Thr Glu Gly 1125 1130 1135	3408
TCT GTT TTT TTG AAC TAT AAA TTA TAT GAA GGA GTA GAA GTC ATT ATA Ser Val Phe Leu Asn Tyr Lys Leu Tyr Glu Gly Val Glu Val Ile Ile 1140 1145 1150	3456
AGA AAA AAT GGT CCT ATA GAT ATA TCT AAT ACA GAT AAT TTT GTT AGA Arg Lys Asn Gly Pro Ile Asp Ile Ser Asn Thr Asp Asn Phe Val Arg 1155 1160 1165	3504
AAA AAC GAT CTA GCA TAC ATT AAT GTA GTA GAT CGT GGT GTA GAA TAT Lys Asn Asp Leu Ala Tyr Ile Asn Val Val Asp Arg Gly Val Glu Tyr 1170 1175 1180	3552
CGG TTA TAT GCT GAT ACA AAA TCA GAG AAA GAG AAA ATA ATA AGA ACA Arg Leu Tyr Ala Asp Thr Lys Ser Glu Lys Glu Lys Ile Ile Arg Thr 1185 1190 1195 1200	3600
TCT AAT CTA AAC GAT AGC TTA GGT CAA ATT ATA GTT ATG GAT TCA ATA Ser Asn Leu Asn Asp Ser Leu Gly Gln Ile Ile Val Met Asp Ser Ile 1205 1210 1215	3648
GGA AAT AAT TGC ACA ATG AAT TTT CAA AAC AAT AAT GGG AGC AAT ATA Gly Asn Asn Cys Thr Met Asn Phe Gln Asn Asn Asn Gly Ser Asn Ile 1220 1225 1230	3696
GGA TTA CTA GGT TTT CAT TCA AAT AAT TTG GTT GCT AGT AGT TGG TAT Gly Leu Leu Gly Phe His Ser Asn Asn Leu Val Ala Ser Ser Trp Tyr 1235 1240 1245	3744
TAT AAC AAT ATA CGA AGA AAT ACT AGC AGT AAT GGA TGC TTT TGG AGT Tyr Asn Asn Ile Arg Arg Asn Thr Ser Ser Asn Gly Cys Phe Trp Ser 1250 1255 1260	3792
TCT ATT TCT AAA GAG AAT GGA TGG AAA GAA TGA Ser Ile Ser Lys Glu Asn Gly Trp Lys Glu 1265 1270	3825

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1274 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

Met Pro Val Ala Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp 1 5 10 15
Asp Thr Ile Leu Tyr Met Gln Ile Pro Tyr Glu Glu Lys Ser Lys Lys 20 25 30
Tyr Tyr Lys Ala Phe Glu Ile Met Arg Asn Val Trp Ile Ile Pro Glu 35 40 45
Arg Asn Thr Ile Gly Thr Asn Pro Ser Asp Phe Asp Pro Pro Ala Ser 50 55 60

Leu Lys Asn Gly Ser Ser Ala Tyr Tyr Asp Pro Asn Tyr Leu Thr Thr  
 65 70 75 80  
 Asp Ala Glu Lys Asp Arg Tyr Leu Lys Thr Thr Ile Lys Leu Phe Lys  
 85 90 95  
 Arg Ile Asn Ser Asn Pro Ala Gly Lys Val Leu Leu Gln Glu Ile Ser  
 100 105 110  
 Tyr Ala Lys Pro Tyr Leu Gly Asn Asp His Thr Pro Ile Asp Glu Phe  
 115 120 125  
 Ser Pro Val Thr Arg Thr Thr Ser Val Asn Ile Lys Leu Ser Thr Asn  
 130 135 140  
 Val Glu Ser Ser Met Leu Leu Asn Leu Leu Val Leu Gly Ala Gly Pro  
 145 150 155 160  
 Asp Ile Phe Glu Ser Cys Cys Tyr Pro Val Arg Lys Leu Ile Asp Pro  
 165 170 175  
 Asp Val Val Tyr Asp Pro Ser Asn Tyr Gly Phe Gly Ser Ile Asn Ile  
 180 185 190  
 Val Thr Phe Ser Pro Glu Tyr Glu Tyr Thr Phe Asn Asp Ile Ser Gly  
 195 200 205  
 Gly His Asn Ser Ser Thr Glu Ser Phe Ile Ala Asp Pro Ala Ile Ser  
 210 215 220  
 Leu Ala His Glu Leu Ile His Ala Leu His Gly Leu Tyr Gly Ala Arg  
 225 230 235 240  
 Gly Val Thr Tyr Glu Glu Thr Ile Glu Val Lys Gln Ala Pro Leu Met  
 245 250 255  
 Ile Ala Glu Lys Pro Ile Arg Leu Glu Glu Phe Leu Thr Phe Gly Gly  
 260 265 270  
 Gln Asp Leu Asn Ile Ile Thr Ser Ala Met Lys Glu Lys Ile Tyr Asn  
 275 280 285  
 Asn Leu Leu Ala Asn Tyr Glu Lys Ile Ala Thr Arg Leu Ser Glu Val  
 290 295 300  
 Asn Ser Ala Pro Pro Glu Tyr Asp Ile Asn Glu Tyr Lys Asp Tyr Phe  
 305 310 315 320  
 Gln Trp Lys Tyr Gly Leu Asp Lys Asn Ala Asp Gly Ser Tyr Thr Val  
 325 330 335  
 Asn Glu Asn Lys Phe Asn Glu Ile Tyr Lys Lys Leu Tyr Ser Phe Thr  
 340 345 350  
 Glu Ser Asp Leu Ala Asn Lys Phe Lys Val Lys Cys Arg Asn Thr Tyr  
 355 360 365  
 Phe Ile Lys Tyr Glu Phe Leu Lys Val Pro Asn Leu Leu Asp Asp Asp  
 370 375 380  
 Ile Tyr Thr Val Ser Glu Gly Phe Asn Ile Gly Asn Leu Ala Val Asn  
 385 390 395 400

Asn Arg Gly Gln Ser Ile Lys Leu Asn Pro Lys Ile Ile Asp Ser Ile  
 405 410 415  
 Pro Asp Lys Gly Leu Val Glu Lys Ile Val Lys Phe Cys Lys Ser Val  
 420 425 430  
 Ile Pro Arg Lys Gly Thr Lys Ala Pro Pro Arg Leu Cys Ile Arg Val  
 435 440 445  
 Asn Asn Ser Glu Leu Phe Phe Val Ala Ser Glu Ser Ser Tyr Asn Glu  
 450 455 460  
 Asn Asp Ile Asn Thr Pro Lys Glu Ile Asp Asp Thr Thr Asn Leu Asn  
 465 470 475 480  
 Asn Asn Tyr Arg Asn Asn Leu Asp Glu Val Ile Leu Asp Tyr Asn Ser  
 485 490 495  
 Gln Thr Ile Pro Gln Ile Ser Asn Arg Thr Leu Asn Thr Leu Val Gln  
 500 505 510  
 Asp Asn Ser Tyr Val Pro Arg Tyr Asp Ser Asn Gly Thr Ser Glu Ile  
 515 520 525  
 Glu Glu Tyr Asp Val Val Asp Phe Asn Val Phe Phe Tyr Leu His Ala  
 530 535 540  
 Gln Lys Val Pro Glu Gly Glu Thr Asn Ile Ser Leu Thr Ser Ser Ile  
 545 550 555 560  
 Asp Thr Ala Leu Leu Glu Glu Ser Lys Asp Ile Phe Phe Ser Ser Glu  
 565 570 575  
 Phe Ile Asp Thr Ile Asn Lys Pro Val Asn Ala Ala Leu Phe Ile Asp  
 580 585 590  
 Trp Ile Ser Lys Val Ile Arg Asp Phe Thr Thr Glu Ala Thr Gln Lys  
 595 600 605  
 Ser Thr Val Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Val  
 610 615 620  
 Gly Leu Ala Leu Asn Ile Ile Ile Glu Ala Glu Lys Gly Asn Phe Glu  
 625 630 635 640  
 Glu Ala Phe Glu Leu Leu Gly Val Gly Ile Leu Leu Glu Phe Val Pro  
 645 650 655  
 Glu Leu Thr Ile Pro Val Ile Leu Val Phe Thr Ile Lys Ser Tyr Ile  
 660 665 670  
 Asp Ser Tyr Glu Asn Lys Asn Lys Ala Ile Lys Ala Ile Asn Asn Ser  
 675 680 685  
 Leu Ile Glu Arg Glu Ala Lys Trp Lys Glu Ile Tyr Ser Trp Ile Val  
 690 695 700  
 Ser Asn Trp Leu Thr Arg Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu  
 705 710 715 720  
 Gln Met Tyr Gln Ala Leu Gln Asn Gln Val Asp Ala Ile Lys Thr Ala  
 725 730 735

Ile Glu Tyr Lys Tyr Asn Asn Tyr Thr Ser Asp Glu Lys Asn Arg Leu  
 740 745 750  
 Glu Ser Glu Tyr Asn Ile Asn Asn Ile Glu Glu Glu Leu Asn Lys Lys  
 755 760 765  
 Val Ser Leu Ala Met Lys Asn Ile Glu Arg Phe Met Thr Glu Ser Ser  
 770 775 780  
 Ile Ser Tyr Leu Met Lys Leu Ile Asn Glu Ala Lys Val Gly Lys Leu  
 785 790 795 800  
 Lys Lys Tyr Asp Asn His Val Lys Ser Asp Leu Leu Asn Tyr Ile Leu  
 805 810 815  
 Asp His Arg Ser Ile Leu Gly Glu Gln Thr Asn Glu Leu Ser Asp Leu  
 820 825 830  
 Val Thr Ser Thr Leu Asn Ser Ser Ile Pro Phe Glu Leu Ser Ser Tyr  
 835 840 845  
 Thr Asn Asp Lys Ile Leu Ile Ile Tyr Phe Asn Arg Leu Tyr Lys Lys  
 850 855 860  
 Ile Lys Asp Ser Ser Ile Leu Asp Met Arg Tyr Glu Asn Asn Lys Phe  
 865 870 875 880  
 Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile Ser Ile Asn Gly Asn Val  
 885 890 895  
 Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe Gly Ile Tyr Asn Ser Arg  
 900 905 910  
 Leu Ser Glu Val Asn Ile Ala Gln Asn Asn Asp Ile Ile Tyr Asn Ser  
 915 920 925  
 Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Lys His  
 930 935 940  
 Tyr Lys Pro Met Asn His Asn Arg Glu Tyr Thr Ile Ile Asn Cys Met  
 945 950 955 960  
 Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser Leu Arg Thr Val Arg Asp  
 965 970 975  
 Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr Ser Gly Asn Lys Glu Asn  
 980 985 990  
 Leu Ile Phe Arg Tyr Glu Glu Leu Asn Arg Ile Ser Asn Tyr Ile Asn  
 995 1000 1005  
 Lys Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Gly Asn Ser Arg  
 1010 1015 1020  
 Ile Tyr Ile Asn Gly Asn Leu Ile Val Glu Lys Ser Ile Ser Asn Leu  
 1025 1030 1035 1040  
 Gly Asp Ile His Val Ser Asp Asn Ile Leu Phe Lys Ile Val Gly Cys  
 1045 1050 1055  
 Asp Asp Glu Thr Tyr Val Gly Ile Arg Tyr Phe Lys Val Phe Asn Thr  
 1060 1065 1070

Glu Leu Asp Lys Thr Glu Ile Glu Thr Leu Tyr Ser Asn Glu Pro Asp  
 1075 1080 1085  
 Pro Ser Ile Leu Lys Asn Tyr Trp Gly Asn Tyr Leu Leu Tyr Asn Lys  
 1090 1095 1100  
 Lys Tyr Tyr Leu Phe Asn Leu Leu Arg Lys Asp Lys Tyr Ile Thr Leu  
 1105 1110 1115 1120  
 Asn Ser Gly Ile Leu Asn Ile Asn Gln Gln Arg Gly Val Thr Glu Gly  
 1125 1130 1135  
 Ser Val Phe Leu Asn Tyr Lys Leu Tyr Glu Gly Val Glu Val Ile Ile  
 1140 1145 1150  
 Arg Lys Asn Gly Pro Ile Asp Ile Ser Asn Thr Asp Asn Phe Val Arg  
 1155 1160 1165  
 Lys Asn Asp Leu Ala Tyr Ile Asn Val Val Asp Arg Gly Val Glu Tyr  
 1170 1175 1180  
 Arg Leu Tyr Ala Asp Thr Lys Ser Glu Lys Glu Lys Ile Ile Arg Thr  
 1185 1190 1195 1200  
 Ser Asn Leu Asn Asp Ser Leu Gly Gln Ile Ile Val Met Asp Ser Ile  
 1205 1210 1215  
 Gly Asn Asn Cys Thr Met Asn Phe Gln Asn Asn Asn Gly Ser Asn Ile  
 1220 1225 1230  
 Gly Leu Leu Gly Phe His Ser Asn Asn Leu Val Ala Ser Ser Trp Tyr  
 1235 1240 1245  
 Tyr Asn Asn Ile Arg Arg Asn Thr Ser Ser Asn Gly Cys Phe Trp Ser  
 1250 1255 1260  
 Ser Ile Ser Lys Glu Asn Gly Trp Lys Glu  
 1265 1270

(2) INFORMATION FOR SEQ ID NO:72:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1460 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 108..1451

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
TTCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATAACC ATG GGC CAT	116
Met Gly His	
1	



CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT His His His His His His His His His Ser Ser Gly His Ile Glu Gly 5 10 15	164
CGT CAT ATG GCT AGC ATG GCT ATT CTA ATT ATA TAT TTT AAT AGA TTA Arg His Met Ala Ser Met Ala Ile Leu Ile Ile Tyr Phe Asn Arg Leu 20 25 30 35	212
TAT AAA AAA ATT AAA GAT AGT TCT ATT TTA GAT ATG CGA TAT GAA AAT Tyr Lys Lys Ile Lys Asp Ser Ser Ile Leu Asp Met Arg Tyr Glu Asn 40 45 50	260
AAT AAA TTT ATA GAT ATC TCT GGA TAT GGT TCA AAT ATA AGC ATT AAT Asn Lys Phe Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile Ser Ile Asn 55 60 65	308
GGA AAC GTA TAT ATT TAT TCA ACA AAT AGA AAT CAA TTT GGA ATA TAT Gly Asn Val Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe Gly Ile Tyr 70 75 80	356
AAT AGT AGG CTT AGT GAA GTT AAT ATA GCT CAA AAT AAT GAT ATT ATA Asn Ser Arg Leu Ser Glu Val Asn Ile Ala Gln Asn Asn Asp Ile Ile 85 90 95	404
TAC AAT AGT AGA TAT CAA AAT TTT AGT ATT AGT TTC TGG GTA AGG ATT Tyr Asn Ser Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp Val Arg Ile 100 105 110 115	452
CCT AAA CAC TAC AAA CCT ATG AAT CAT AAT CGG GAA TAC ACT ATA ATA Pro Lys His Tyr Lys Pro Met Asn His Asn Arg Glu Tyr Thr Ile Ile 120 125 130	500
AAT TGT ATG GGG AAT AAT AAT TCG GGA TGG AAA ATA TCA CTT AGA ACT Asn Cys Met Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser Leu Arg Thr 135 140 145	548
GTT AGA GAT TGT GAA ATA ATT TGG ACT TTA CAA GAT ACT TCT GGA AAT Val Arg Asp Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr Ser Gly Asn 150 155 160	596
AAG GAA AAT TTA ATT TTT AGG TAT GAA GAA CTT AAT AGG ATA TCT AAT Lys Glu Asn Leu Ile Phe Arg Tyr Glu Glu Leu Asn Arg Ile Ser Asn 165 170 175	644
TAT ATA AAT AAA TGG ATT TTT GTA ACT ATT ACT AAT AAT AGA TTA GGC Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Gly 180 185 190 195	692
AAT TCT AGA ATT TAC ATC AAT GGA AAT TTA ATA GTT GAA AAA TCA ATT Asn Ser Arg Ile Tyr Ile Asn Gly Asn Leu Ile Val Glu Lys Ser Ile 200 205 210	740
TCG AAT TTA GGT GAT ATT CAT GTT AGT GAT AAT ATA TTA TTT AAA ATT Ser Asn Leu Gly Asp Ile His Val Ser Asp Asn Ile Leu Phe Lys Ile 215 220 225	788
GTT GGT TGT GAT GAT GAA ACG TAT GTT GGT ATA AGA TAT TTT AAA GTT Val Gly Cys Asp Asp Glu Thr Tyr Val Gly Ile Arg Tyr Phe Lys Val 230 235 240	836

TTT AAT ACG GAA TTA GAT AAA ACA GAA ATT GAG ACT TTA TAT AGT AAT Phe Asn Thr Glu Leu Asp Lys Thr Glu Ile Glu Thr Leu Tyr Ser Asn 245 250 255	884
GAG CCA GAT CCA AGT ATC TTA AAA AAC TAT TGG GGA AAT TAT TTG CTA Glu Pro Asp Pro Ser Ile Leu Lys Asn Tyr Trp Gly Asn Tyr Leu Leu 260 265 270 275	932
TAT AAT AAA AAA TAT TAT TTA TTC AAT TTA CTA AGA AAA GAT AAG TAT Tyr Asn Lys Lys Tyr Tyr Leu Phe Asn Leu Arg Lys Asp Lys Tyr 280 285 290	980
ATT ACT CTG AAT TCA GGC ATT TTA AAT ATT AAT CAA CAA AGA GGT GTT Ile Thr Leu Asn Ser Gly Ile Leu Asn Ile Asn Gln Gln Arg Gly Val 295 300 305	1028
ACT GAA GGC TCT GTT TTT TTG AAC TAT AAA TTA TAT GAA GGA GTA GAA Thr Glu Gly Ser Val Phe Leu Asn Tyr Lys Leu Tyr Glu Gly Val Glu 310 315 320	1076
GTC ATT ATA AGA AAA AAT GGT CCT ATA GAT ATA TCT AAT ACA GAT AAT Val Ile Ile Arg Lys Asn Gly Pro Ile Asp Ile Ser Asn Thr Asp Asn 325 330 335	1124
TTT GTT AGA AAA AAC GAT CTA GCA TAC ATT AAT GTA GTA GAT CGT GGT Phe Val Arg Lys Asn Asp Leu Ala Tyr Ile Asn Val Val Asp Arg Gly 340 345 350 355	1172
GTA GAA TAT CGG TTA TAT GCT GAT ACA AAA TCA GAG AAA GAG AAA ATA Val Glu Tyr Arg Leu Tyr Ala Asp Thr Lys Ser Glu Lys Glu Lys Ile 360 365 370	1220
ATA AGA ACA TCT AAT CTA AAC GAT AGC TTA GGT CAA ATT ATA GTT ATG Ile Arg Thr Ser Asn Leu Asn Asp Ser Leu Gly Gln Ile Ile Val Met 375 380 385	1268
GAT TCA ATA GGA AAT AAT TGC ACA ATG AAT TTT CAA AAC AAT AAT GGG Asp Ser Ile Gly Asn Asn Cys Thr Met Asn Phe Gln Asn Asn Asn Gly 390 395 400	1316
AGC AAT ATA GGA TTA CTA GGT TTT CAT TCA AAT AAT TTG GTT GCT AGT Ser Asn Ile Gly Leu Leu Gly Phe His Ser Asn Asn Leu Val Ala Ser 405 410 415	1364
AGT TGG TAT TAT AAC AAT ATA CGA AGA AAT ACT AGC AGT AAT GGA TGC Ser Trp Tyr Tyr Asn Asn Ile Arg Arg Asn Thr Ser Ser Asn Gly Cys 420 425 430 435	1412
TTT TGG AGT TCT ATT TCT AAA GAG AAT GGA TGG AAA GAA TGAAAGCTT Phe Trp Ser Ser Ile Ser Lys Glu Asn Gly Trp Lys Glu 440 445	1460

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 448 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

Met Gly His His His His His His His His His His Ser Ser Gly His  
1 5 10 15  
Ile Glu Gly Arg His Met Ala Ser Met Ala Ile Leu Ile Ile Tyr Phe  
20 25 30  
Asn Arg Leu Tyr Lys Lys Ile Lys Asp Ser Ser Ile Leu Asp Met Arg  
35 40 45  
Tyr Glu Asn Asn Lys Phe Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile  
50 55 60  
Ser Ile Asn Gly Asn Val Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe  
65 70 75 80  
Gly Ile Tyr Asn Ser Arg Leu Ser Glu Val Asn Ile Ala Gln Asn Asn  
85 90 95  
Asp Ile Ile Tyr Asn Ser Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp  
100 105 110  
Val Arg Ile Pro Lys His Tyr Lys Pro Met Asn His Asn Arg Glu Tyr  
115 120 125  
Thr Ile Ile Asn Cys Met Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser  
130 135 140  
Leu Arg Thr Val Arg Asp Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr  
145 150 155 160  
Ser Gly Asn Lys Glu Asn Leu Ile Phe Arg Tyr Glu Glu Leu Asn Arg  
165 170 175  
Ile Ser Asn Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile Thr Asn Asn  
180 185 190  
Arg Leu Gly Asn Ser Arg Ile Tyr Ile Asn Gly Asn Leu Ile Val Glu  
195 200 205  
Lys Ser Ile Ser Asn Leu Gly Asp Ile His Val Ser Asp Asn Ile Leu  
210 215 220  
Phe Lys Ile Val Gly Cys Asp Asp Glu Thr Tyr Val Gly Ile Arg Tyr  
225 230 235 240  
Phe Lys Val Phe Asn Thr Glu Leu Asp Lys Thr Glu Ile Glu Thr Leu  
245 250 255  
Tyr Ser Asn Glu Pro Asp Pro Ser Ile Leu Lys Asn Tyr Trp Gly Asn  
260 265 270  
Tyr Leu Leu Tyr Asn Lys Lys Tyr Tyr Leu Phe Asn Leu Leu Arg Lys  
275 280 285  
Asp Lys Tyr Ile Thr Leu Asn Ser Gly Ile Leu Asn Ile Asn Gln Gln  
290 295 300  
Arg Gly Val Thr Glu Gly Ser Val Phe Leu Asn Tyr Lys Leu Tyr Glu  
305 310 315 320



(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3894 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..3891

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

ATG CCA GTT AAT ATA AAA AAC TTT AAT TAT AAT GAC CCT ATT AAT AAT	48
Met Pro Val Asn Ile Lys Asn Phe Asn Tyr Asn Asp Pro Ile Asn Asn	
1 5 10 15	
GAT GAC ATT ATT ATG ATG GAA CCA TTC AAT GAC CCA GGG CCA GGA ACA	96
Asp Asp Ile Ile Met Met Glu Pro Phe Asn Asp Pro Gly Pro Gly Thr	
20 25 30	
TAT TAT AAA GCT TTT AGG ATT ATA GAT CGT ATT TGG ATA GTA CCA GAA	144
Tyr Tyr Lys Ala Phe Arg Ile Ile Asp Arg Ile Trp Ile Val Pro Glu	
35 40 45	
AGG TTT ACT TAT GGA TTT CAA CCT GAC CAA TTT AAT GCC AGT ACA GGA	192
Arg Phe Thr Tyr Gly Phe Gln Pro Asp Gln Phe Asn Ala Ser Thr Gly	
50 55 60	
GTT TTT AGT AAA GAT GTC TAC GAA TAT TAC GAT CCA ACT TAT TTA AAA	240
Val Phe Ser Lys Asp Val Tyr Glu Tyr Tyr Asp Pro Thr Tyr Leu Lys	
65 70 75 80	
ACC GAT GCT GAA AAA GAT AAA TTT TTA AAA ACA ATG ATT AAA TTA TTT	288
Thr Asp Ala Glu Lys Asp Lys Phe Leu Lys Thr Met Ile Lys Leu Phe	
85 90 95	
AAT AGA ATT AAT TCA AAA CCA TCA GGA CAG AGA TTA CTG GAT ATG ATA	336
Asn Arg Ile Asn Ser Lys Pro Ser Gly Gln Arg Leu Leu Asp Met Ile	
100 105 110	
GTA GAT GCT ATA CCT TAT CTT GGA AAT GCA TCT ACA CCG CCC GAC AAA	384
Val Asp Ala Ile Pro Tyr Leu Gly Asn Ala Ser Thr Pro Pro Asp Lys	
115 120 125	
TTT GCA GCA AAT GTT GCA AAT GTA TCT ATT AAT AAA AAA ATT ATC CAA	432
Phe Ala Ala Asn Val Ala Asn Val Ser Ile Asn Lys Lys Ile Ile Gln	
130 135 140	
CCT GGA GCT GAA GAT CAA ATA AAA GGT TTA ATG ACA AAT TTA ATA ATA	480
Pro Gly Ala Glu Asp Gln Ile Lys Gly Leu Met Thr Asn Leu Ile Ile	
145 150 155 160	
TTT GGA CCA GGA CCA GTT CTA AGT GAT AAT TTT ACT GAT AGT ATG ATT	528
Phe Gly Pro Gly Pro Val Leu Ser Asp Asn Phe Thr Asp Ser Met Ile	
165 170 175	

ATG AAT GGC CAT TCC CCA ATA TCA GAA GGA TTT GGT GCA AGA ATG ATG Met Asn Gly His Ser Pro Ile Ser Glu Gly Phe Gly Ala Arg Met Met 180 185 190	576
ATA AGA TTT TGT CCT AGT TGT TTA AAT GTA TTT AAT AAT GTT CAG GAA Ile Arg Phe Cys Pro Ser Cys Leu Asn Val Phe Asn Asn Val Gln Glu 195 200 205	624
AAT AAA GAT ACA TCT ATA TTT AGT AGA CGC GCG TAT TTT GCA GAT CCA Asn Lys Asp Thr Ser Ile Phe Ser Arg Arg Ala Tyr Phe Ala Asp Pro 210 215 220	672
GCT CTA ACG TTA ATG CAT GAA CTT ATA CAT GTG TTA CAT GGA TTA TAT Ala Leu Thr Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr 225 230 235 240	720
GGA ATT AAG ATA AGT AAT TTA CCA ATT ACT CCA AAT ACA AAA GAA TTT Gly Ile Lys Ile Ser Asn Leu Pro Ile Thr Pro Asn Thr Lys Glu Phe 245 250 255	768
TTC ATG CAA CAT AGC GAT CCT GTA CAA GCA GAA GAA CTA TAT ACA TTC Phe Met Gln His Ser Asp Pro Val Gln Ala Glu Glu Leu Tyr Thr Phe 260 265 270	816
GGA GGA CAT GAT CCT AGT GTT ATA AGT CCT TCT ACG GAT ATG AAT ATT Gly Gly His Asp Pro Ser Val Ile Ser Pro Ser Thr Asp Met Asn Ile 275 280 285	864
TAT AAT AAA GCG TTA CAA AAT TTT CAA GAT ATA GCT AAT AGG CTT AAT Tyr Asn Lys Ala Leu Gln Asn Phe Gln Asp Ile Ala Asn Arg Leu Asn 290 295 300	912
ATT GTT TCA AGT GCC CAA GGG AGT GGA ATT GAT ATT TCC TTA TAT AAA Ile Val Ser Ser Ala Gln Gly Ser Gly Ile Asp Ile Ser Leu Tyr Lys 305 310 315 320	960
CAA ATA TAT AAA AAT AAA TAT GAT TTT GTT GAA GAT CCT AAT GGA AAA Gln Ile Tyr Lys Asn Lys Tyr Asp Phe Val Glu Asp Pro Asn Gly Lys 325 330 335	1008
TAT AGT GTA GAT AAG GAT AAG TTT GAT AAA TTA TAT AAG GCC TTA ATG Tyr Ser Val Asp Lys Asp Lys Phe Asp Lys Leu Tyr Lys Ala Leu Met 340 345 350	1056
TTT GGC TTT ACT GAA ACT AAT CTA GCT GGT GAA TAT GGA ATA AAA ACT Phe Gly Phe Thr Glu Thr Asn Leu Ala Gly Glu Tyr Gly Ile Lys Thr 355 360 365	1104
AGG TAT TCT TAT TTT AGT GAA TAT TTG CCA CCG ATA AAA ACT GAA AAA Arg Tyr Ser Tyr Phe Ser Glu Tyr Leu Pro Pro Ile Lys Thr Glu Lys 370 375 380	1152
TTG TTA GAC AAT ACA ATT TAT ACT CAA AAT GAA GGC TTT AAC ATA GCT Leu Leu Asp Asn Thr Ile Tyr Thr Gln Asn Glu Gly Phe Asn Ile Ala 385 390 395 400	1200
AGT AAA AAT CTC AAA ACG GAA TTT AAT GGT CAG AAT AAG GCG GTA AAT Ser Lys Asn Leu Lys Thr Glu Phe Asn Gly Gln Asn Lys Ala Val Asn 405 410 415	1248

AAA GAG GCT TAT GAA GAA ATC AGC CTA GAA CAT CTC GTT ATA TAT AGA Lys Glu Ala Tyr Glu Glu Ile Ser Leu Glu His Leu Val Ile Tyr Arg 420 425 430	1296
ATA GCA ATG TGC AAG CCT GTA ATG TAC AAA AAT ACC GGT AAA TCT GAA Ile Ala Met Cys Lys Pro Val Met Tyr Lys Asn Thr Gly Lys Ser Glu 435 440 445	1344
CAG TGT ATT ATT GTT AAT AAT GAG GAT TTA TTT TTC ATA GCT AAT AAA Gln Cys Ile Ile Val Asn Asn Glu Asp Leu Phe Ile Ala Asn Lys 450 455 460	1392
GAT AGT TTT TCA AAA GAT TTA GCT AAA GCA GAA ACT ATA GCA TAT AAT Asp Ser Phe Ser Lys Asp Leu Ala Lys Ala Glu Thr Ile Ala Tyr Asn 465 470 475 480	1440
ACA CAA AAT AAT ACT ATA GAA AAT AAT TTT TCT ATA GAT CAG TTG ATT Thr Gln Asn Asn Thr Ile Glu Asn Asn Phe Ser Ile Asp Gln Leu Ile 485 490 495	1488
TTA GAT AAT GAT TTA AGC AGT GGC ATA GAC TTA CCA AAT GAA AAC ACA Leu Asp Asn Asp Leu Ser Ser Gly Ile Asp Leu Pro Asn Glu Asn Thr 500 505 510	1536
GAA CCA TTT ACA AAT TTT GAC GAC ATA GAT ATC CCT GTG TAT ATT AAA Glu Pro Phe Thr Asn Phe Asp Asp Ile Asp Ile Pro Val Tyr Ile Lys 515 520 525	1584
CAA TCT GCT TTA AAA AAA ATT TTT GTG GAT GGA GAT AGC CTT TTT GAA Gln Ser Ala Leu Lys Lys Ile Phe Val Asp Gly Asp Ser Leu Phe Glu 530 535 540	1632
TAT TTA CAT GCT CAA ACA TTT CCT TCT AAT ATA GAA AAT CTA CAA CTA Tyr Leu His Ala Gln Thr Phe Pro Ser Asn Ile Glu Asn Leu Gln Leu 545 550 555 560	1680
ACG AAT TCA TTA AAT GAT GCT TTA AGA AAT AAT AAT AAA GTC TAT ACT Thr Asn Ser Leu Asn Asp Ala Leu Arg Asn Asn Asn Lys Val Tyr Thr 565 570 575	1728
TTT TTT TCT ACA AAC CTT GTT GAA AAA GCT AAT ACA GTT GTA GGT GCT Phe Phe Ser Thr Asn Leu Val Glu Lys Ala Asn Thr Val Val Gly Ala 580 585 590	1776
TCA CTT TTT GTA AAC TGG GTA AAA GGA GTA ATA GAT GAT TTT ACA TCT Ser Leu Phe Val Asn Trp Val Lys Gly Val Ile Asp Asp Phe Thr Ser 595 600 605	1824
GAA TCC ACA CAA AAA AGT ACT ATA GAT AAA GTT TCA GAT GTA TCC ATA Glu Ser Thr Gln Lys Ser Thr Ile Asp Lys Val Ser Asp Val Ser Ile 610 615 620	1872
ATT ATT CCC TAT ATA GGA CCT GCT TTG AAT GTA GGA AAT GAA ACA GCT Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Val Gly Asn Glu Thr Ala 625 630 635 640	1920
AAA GAA AAT TTT AAA AAT GCT TTT GAA ATA GGT GGA GCC GCT ATC TTA Lys Glu Asn Phe Lys Asn Ala Phe Glu Ile Gly Gly Ala Ala Ile Leu 645 650 655	1968

ATG GAG TTT ATT CCA GAA CTT ATT GTA CCT ATA GTT GGA TTT TTT ACA Met Glu Phe Ile Pro Glu Leu Ile Val Pro Ile Val Gly Phe Phe Thr 660 665 670	2016
TTA GAA TCA TAT GTA GGA AAT AAA GGG CAT ATT ATT ATG ACG ATA TCC Leu Glu Ser Tyr Val Gly Asn Lys Gly His Ile Ile Met Thr Ile Ser 675 680 685	2064
AAT GCT TTA AAG AAA AGG GAT CAA AAA TGG ACA GAT ATG TAT GGT TTG Asn Ala Leu Lys Lys Arg Asp Gln Lys Trp Thr Asp Met Tyr Gly Leu 690 695 700	2112
ATA GTA TCG CAG TGG CTC TCA ACG GTT AAT ACT CAA TTT TAT ACA ATA Ile Val Ser Gln Trp Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile 705 710 715 720	2160
AAA GAA AGA ATG TAC AAT GCT TTA AAT AAT CAA TCA CAA GCA ATA GAA Lys Glu Arg Met Tyr Asn Ala Leu Asn Asn Gln Ser Gln Ala Ile Glu 725 730 735	2208
AAA ATA ATA GAA GAT CAA TAT AAT AGA TAT AGT GAA GAA GAT AAA ATG Lys Ile Ile Glu Asp Gln Tyr Asn Arg Tyr Ser Glu Glu Asp Lys Met 740 745 750	2256
AAT ATT AAC ATT GAT TTT AAT GAT ATA GAT TTT AAA CTT AAT CAA AGT Asn Ile Asn Ile Asp Phe Asn Asp Ile Asp Phe Lys Leu Asn Gln Ser 755 760 765	2304
ATA AAT TTA GCA ATA AAC AAT ATA GAT GAT TTT ATA AAC CAA TGT TCT Ile Asn Leu Ala Ile Asn Asn Ile Asp Asp Phe Ile Asn Gln Cys Ser 770 775 780	2352
ATA TCA TAT CTA ATG AAT AGA ATG ATT CCA TTA GCT GTA AAA AAG TTA Ile Ser Tyr Leu Met Asn Arg Met Ile Pro Leu Ala Val Lys Lys Leu 785 790 795 800	2400
AAA GAC TTT GAT GAT AAT CTT AAG AGA GAT TTA TTG GAG TAT ATA GAT Lys Asp Phe Asp Asp Asn Leu Lys Arg Asp Leu Leu Glu Tyr Ile Asp 805 810 815	2448
ACA AAT GAA CTA TAT TTA CTT GAT GAA GTA AAT ATT CTA AAA TCA AAA Thr Asn Glu Leu Tyr Leu Leu Asp Glu Val Asn Ile Leu Lys Ser Lys 820 825 830	2496
GTA AAT AGA CAC CTA AAA GAC AGT ATA CCA TTT GAT CTT TCA CTA TAT Val Asn Arg His Leu Lys Asp Ser Ile Pro Phe Asp Leu Ser Leu Tyr 835 840 845	2544
ACC AAG GAC ACA ATT TTA ATA CAA GTT TTT AAT AAT TAT ATT AGT AAT Thr Lys Asp Thr Ile Leu Ile Gln Val Phe Asn Asn Tyr Ile Ser Asn 850 855 860	2592
ATT AGT AGT AAT GCT ATT TTA AGT TTA AGT TAT AGA GGT GGG CGT TTA Ile Ser Ser Asn Ala Ile Leu Ser Leu Ser Tyr Arg Gly Gly Arg Leu 865 870 875 880	2640
ATA GAT TCA TCT GGA TAT GGT GCA ACT ATG AAT GTA GGT TCA GAT GTT Ile Asp Ser Ser Gly Tyr Gly Ala Thr Met Asn Val Gly Ser Asp Val 885 890 895	2688



ATC TTT AAT GAT ATA GGA AAT GGT CAA TTT AAA TTA AAT AAT TCT GAA Ile Phe Asn Asp Ile Gly Asn Gly Gln Phe Lys Leu Asn Asn Ser Glu 900 905 910	2736
AAT AGT AAT ATT ACG GCA CAT CAA AGT AAA TTC GTT GTA TAT GAT AGT Asn Ser Asn Ile Thr Ala His Gln Ser Lys Phe Val Val Tyr Asp Ser 915 920 925	2784
ATG TTT GAT AAT TTT AGC ATT AAC TTT TGG GTA AGG ACT CCT AAA TAT Met Phe Asp Asn Phe Ser Ile Asn Phe Trp Val Arg Thr Pro Lys Tyr 930 935 940	2832
AAT AAT AAT GAT ATA CAA ACT TAT CTT CAA AAT GAG TAT ACA ATA ATT Asn Asn Asn Asp Ile Gln Thr Tyr Leu Gln Asn Glu Tyr Thr Ile Ile 945 950 955 960	2880
AGT TGT ATA AAA AAT GAC TCA GGA TGG AAA GTA TCT ATT AAG GGA AAT Ser Cys Ile Lys Asn Asp Ser Gly Trp Lys Val Ser Ile Lys Gly Asn 965 970 975	2928
AGA ATA ATA TGG ACA TTA ATA GAT GTT AAT GCA AAA TCT AAA TCA ATA Arg Ile Ile Trp Thr Leu Ile Asp Val Asn Ala Lys Ser Lys Ser Ile 980 985 990	2976
TTT TTC GAA TAT AGT ATA AAA GAT AAT ATA TCA GAT TAT ATA AAT AAA Phe Phe Glu Tyr Ser Ile Lys Asp Asn Ile Ser Asp Tyr Ile Asn Lys 995 1000 1005	3024
TGG TTT TCC ATA ACT ATT ACT AAT GAT AGA TTA GGT AAC GCA AAT ATT Trp Phe Ser Ile Thr Ile Thr Asn Asp Arg Leu Gly Asn Ala Asn Ile 1010 1015 1020	3072
TAT ATA AAT GGA AGT TTG AAA AAA AGT GAA AAA ATT TTA AAC TTA GAT Tyr Ile Asn Gly Ser Leu Lys Lys Ser Glu Lys Ile Leu Asn Leu Asp 1025 1030 1035 1040	3120
AGA ATT AAT TCT AGT AAT GAT ATA GAC TTC AAA TTA ATT AAT TGT ACA Arg Ile Asn Ser Ser Asn Asp Ile Asp Phe Lys Leu Ile Asn Cys Thr 1045 1050 1055	3168
GAT ACT ACT AAA TTT GTT TGG ATT AAG GAT TTT AAT ATT TTT GGT AGA Asp Thr Thr Lys Phe Val Trp Ile Lys Asp Phe Asn Ile Phe Gly Arg 1060 1065 1070	3216
GAA TTA AAT GCT ACA GAA GTA TCT TCA CTA TAT TGG ATT CAA TCA TCT Glu Leu Asn Ala Thr Glu Val Ser Ser Leu Tyr Trp Ile Gln Ser Ser 1075 1080 1085	3264
ACA AAT ACT TTA AAA GAT TTT TGG GGG AAT CCT TTA AGA TAC GAT ACA Thr Asn Thr Leu Lys Asp Phe Trp Gly Asn Pro Leu Arg Tyr Asp Thr 1090 1095 1100	3312
CAA TAC TAT CTG TTT AAT CAA GGT ATG CAA AAT ATC TAT ATA AAG TAT Gln Tyr Tyr Leu Phe Asn Gln Gly Met Gln Asn Ile Tyr Ile Lys Tyr 1105 1110 1115 1120	3360
TTT AGT AAA GCT TCT ATG GGG GAA ACT GCA CCA CGT ACA AAC TTT AAT Phe Ser Lys Ala Ser Met Gly Glu Thr Ala Pro Arg Thr Asn Phe Asn 1125 1130 1135	3408

AAT GCA GCA ATA AAT TAT CAA AAT TTA TAT CTT GGT TTA CGA TTT ATT Asn Ala Ala Ile Asn Tyr Gln Asn Leu Tyr Leu Gly Leu Arg Phe Ile 1140 1145 1150	3456
ATA AAA AAA GCA TCA AAT TCT CGG AAT ATA AAT AAT GAT AAT ATA GTC Ile Lys Lys Ala Ser Asn Ser Arg Asn Ile Asn Asn Asp Asn Ile Val 1155 1160 1165	3504
AGA GAA GGA GAT TAT ATA TAT CTT AAT ATT GAT AAT ATT TCT GAT GAA Arg Glu Gly Asp Tyr Ile Tyr Leu Asn Ile Asp Asn Ile Ser Asp Glu 1170 1175 1180	3552
TCT TAC AGA GTA TAT GTT TTG GTG AAT TCT AAA GAA ATT CAA ACT CAA Ser Tyr Arg Val Tyr Val Leu Val Asn Ser Lys Glu Ile Gln Thr Gln 1185 1190 1195 1200	3600
TTA TTT TTA GCA CCC ATA AAT GAT GAT CCT ACG TTC TAT GAT GTA CTA Leu Phe Leu Ala Pro Ile Asn Asp Asp Pro Thr Phe Tyr Asp Val Leu 1205 1210 1215	3648
CAA ATA AAA AAA TAT TAT GAA AAA ACA ACA TAT AAT TGT CAG ATA CTT Gln Ile Lys Lys Tyr Tyr Glu Lys Thr Thr Tyr Asn Cys Gln Ile Leu 1220 1225 1230	3696
TGC GAA AAA GAT ACT AAA ACA TTT GGG CTG TTT GGA ATT GGT AAA TTT Cys Glu Lys Asp Thr Lys Thr Phe Gly Leu Phe Gly Ile Gly Lys Phe 1235 1240 1245	3744
GTT AAA GAT TAT GGA TAT GTT TGG GAT ACC TAT GAT AAT TAT TTT TGC Val Lys Asp Tyr Gly Tyr Val Trp Asp Thr Tyr Asp Asn Tyr Phe Cys 1250 1255 1260	3792
ATA AGT CAG TGG TAT CTC AGA AGA ATA TCT GAA AAT ATA AAT AAA TTA Ile Ser Gln Trp Tyr Leu Arg Arg Ile Ser Glu Asn Ile Asn Lys Leu 1265 1270 1275 1280	3840
AGG TTG GGA TGT AAT TGG CAA TTC ATT CCC GTG GAT GAA GGA TGG ACA Arg Leu Gly Cys Asn Trp Gln Phe Ile Pro Val Asp Glu Gly Trp Thr 1285 1290 1295	3888
GAA TAA Glu	3894

(2) INFORMATION FOR SEQ ID NO:77:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1297 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

Met Pro Val Asn Ile Lys Asn Phe Asn Tyr Asn Asp Pro Ile Asn Asn 1 5 10 15
Asp Asp Ile Ile Met Met Glu Pro Phe Asn Asp Pro Gly Pro Gly Thr 20 25 30
Tyr Tyr Lys Ala Phe Arg Ile Ile Asp Arg Ile Trp Ile Val Pro Glu 35 40 45

Arg Phe Thr Tyr Gly Phe Gln Pro Asp Gln Phe Asn Ala Ser Thr Gly  
 50 55 60  
 Val Phe Ser Lys Asp Val Tyr Glu Tyr Tyr Asp Pro Thr Tyr Leu Lys  
 65 70 75 80  
 Thr Asp Ala Glu Lys Asp Lys Phe Leu Lys Thr Met Ile Lys Leu Phe  
 85 90 95  
 Asn Arg Ile Asn Ser Lys Pro Ser Gly Gln Arg Leu Leu Asp Met Ile  
 100 105 110  
 Val Asp Ala Ile Pro Tyr Leu Gly Asn Ala Ser Thr Pro Pro Asp Lys  
 115 120 125  
 Phe Ala Ala Asn Val Ala Asn Val Ser Ile Asn Lys Lys Ile Ile Gln  
 130 135 140  
 Pro Gly Ala Glu Asp Gln Ile Lys Gly Leu Met Thr Asn Leu Ile Ile  
 145 150 155 160  
 Phe Gly Pro Gly Pro Val Leu Ser Asp Asn Phe Thr Asp Ser Met Ile  
 165 170 175  
 Met Asn Gly His Ser Pro Ile Ser Glu Gly Phe Gly Ala Arg Met Met  
 180 185 190  
 Ile Arg Phe Cys Pro Ser Cys Leu Asn Val Phe Asn Asn Val Gln Glu  
 195 200 205  
 Asn Lys Asp Thr Ser Ile Phe Ser Arg Arg Ala Tyr Phe Ala Asp Pro  
 210 215 220  
 Ala Leu Thr Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr  
 225 230 235 240  
 Gly Ile Lys Ile Ser Asn Leu Pro Ile Thr Pro Asn Thr Lys Glu Phe  
 245 250 255  
 Phe Met Gln His Ser Asp Pro Val Gln Ala Glu Glu Leu Tyr Thr Phe  
 260 265 270  
 Gly Gly His Asp Pro Ser Val Ile Ser Pro Ser Thr Asp Met Asn Ile  
 275 280 285  
 Tyr Asn Lys Ala Leu Gln Asn Phe Gln Asp Ile Ala Asn Arg Leu Asn  
 290 295 300  
 Ile Val Ser Ser Ala Gln Gly Ser Gly Ile Asp Ile Ser Leu Tyr Lys  
 305 310 315 320  
 Gln Ile Tyr Lys Asn Lys Tyr Asp Phe Val Glu Asp Pro Asn Gly Lys  
 325 330 335  
 Tyr Ser Val Asp Lys Asp Lys Phe Asp Lys Leu Tyr Lys Ala Leu Met  
 340 345 350  
 Phe Gly Phe Thr Glu Thr Asn Leu Ala Gly Glu Tyr Gly Ile Lys Thr  
 355 360 365  
 Arg Tyr Ser Tyr Phe Ser Glu Tyr Leu Pro Pro Ile Lys Thr Glu Lys  
 370 375 380

Leu Leu Asp Asn Thr Ile Tyr Thr Gln Asn Glu Gly Phe Asn Ile Ala  
 385 390 395 400  
 Ser Lys Asn Leu Lys Thr Glu Phe Asn Gly Gln Asn Lys Ala Val Asn  
 405 410 415  
 Lys Glu Ala Tyr Glu Glu Ile Ser Leu Glu His Leu Val Ile Tyr Arg  
 420 425 430  
 Ile Ala Met Cys Lys Pro Val Met Tyr Lys Asn Thr Gly Lys Ser Glu  
 435 440 445  
 Gln Cys Ile Ile Val Asn Asn Glu Asp Leu Phe Phe Ile Ala Asn Lys  
 450 455 460  
 Asp Ser Phe Ser Lys Asp Leu Ala Lys Ala Glu Thr Ile Ala Tyr Asn  
 465 470 475 480  
 Thr Gln Asn Asn Thr Ile Glu Asn Asn Phe Ser Ile Asp Gln Leu Ile  
 485 490 495  
 Leu Asp Asn Asp Leu Ser Ser Gly Ile Asp Leu Pro Asn Glu Asn Thr  
 500 505 510  
 Glu Pro Phe Thr Asn Phe Asp Asp Ile Asp Ile Pro Val Tyr Ile Lys  
 515 520 525  
 Gln Ser Ala Leu Lys Lys Ile Phe Val Asp Gly Asp Ser Leu Phe Glu  
 530 535 540  
 Tyr Leu His Ala Gln Thr Phe Pro Ser Asn Ile Glu Asn Leu Gln Leu  
 545 550 555 560  
 Thr Asn Ser Leu Asn Asp Ala Leu Arg Asn Asn Asn Lys Val Tyr Thr  
 565 570 575  
 Phe Phe Ser Thr Asn Leu Val Glu Lys Ala Asn Thr Val Val Gly Ala  
 580 585 590  
 Ser Leu Phe Val Asn Trp Val Lys Gly Val Ile Asp Asp Phe Thr Ser  
 595 600 605  
 Glu Ser Thr Gln Lys Ser Thr Ile Asp Lys Val Ser Asp Val Ser Ile  
 610 615 620  
 Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Val Gly Asn Glu Thr Ala  
 625 630 635 640  
 Lys Glu Asn Phe Lys Asn Ala Phe Glu Ile Gly Gly Ala Ala Ile Leu  
 645 650 655  
 Met Glu Phe Ile Pro Glu Leu Ile Val Pro Ile Val Gly Phe Phe Thr  
 660 665 670  
 Leu Glu Ser Tyr Val Gly Asn Lys Gly His Ile Ile Met Thr Ile Ser  
 675 680 685  
 Asn Ala Leu Lys Lys Arg Asp Gln Lys Trp Thr Asp Met Tyr Gly Leu  
 690 695 700  
 Ile Val Ser Gln Trp Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile  
 705 710 715 720

Lys Glu Arg Met Tyr Asn Ala Leu Asn Asn Gln Ser Gln Ala Ile Glu  
 725 730 735  
 Lys Ile Ile Glu Asp Gln Tyr Asn Arg Tyr Ser Glu Glu Asp Lys Met  
 740 745 750  
 Asn Ile Asn Ile Asp Phe Asn Asp Ile Asp Phe Lys Leu Asn Gln Ser  
 755 760 765  
 Ile Asn Leu Ala Ile Asn Asn Ile Asp Asp Phe Ile Asn Gln Cys Ser  
 770 775 780  
 Ile Ser Tyr Leu Met Asn Arg Met Ile Pro Leu Ala Val Lys Lys Leu  
 785 790 795 800  
 Lys Asp Phe Asp Asp Asn Leu Lys Arg Asp Leu Leu Glu Tyr Ile Asp  
 805 810 815  
 Thr Asn Glu Leu Tyr Leu Leu Asp Glu Val Asn Ile Leu Lys Ser Lys  
 820 825 830  
 Val Asn Arg His Leu Lys Asp Ser Ile Pro Phe Asp Leu Ser Leu Tyr  
 835 840 845  
 Thr Lys Asp Thr Ile Leu Ile Gln Val Phe Asn Asn Tyr Ile Ser Asn  
 850 855 860  
 Ile Ser Ser Asn Ala Ile Leu Ser Leu Ser Tyr Arg Gly Gly Arg Leu  
 865 870 875 880  
 Ile Asp Ser Ser Gly Tyr Gly Ala Thr Met Asn Val Gly Ser Asp Val  
 885 890 895  
 Ile Phe Asn Asp Ile Gly Asn Gly Gln Phe Lys Leu Asn Asn Ser Glu  
 900 905 910  
 Asn Ser Asn Ile Thr Ala His Gln Ser Lys Phe Val Val Tyr Asp Ser  
 915 920 925  
 Met Phe Asp Asn Phe Ser Ile Asn Phe Trp Val Arg Thr Pro Lys Tyr  
 930 935 940  
 Asn Asn Asn Asp Ile Gln Thr Tyr Leu Gln Asn Glu Tyr Thr Ile Ile  
 945 950 955 960  
 Ser Cys Ile Lys Asn Asp Ser Gly Trp Lys Val Ser Ile Lys Gly Asn  
 965 970 975  
 Arg Ile Ile Trp Thr Leu Ile Asp Val Asn Ala Lys Ser Lys Ser Ile  
 980 985 990  
 Phe Phe Glu Tyr Ser Ile Lys Asp Asn Ile Ser Asp Tyr Ile Asn Lys  
 995 1000 1005  
 Trp Phe Ser Ile Thr Ile Thr Asn Asp Arg Leu Gly Asn Ala Asn Ile  
 1010 1015 1020  
 Tyr Ile Asn Gly Ser Leu Lys Lys Ser Glu Lys Ile Leu Asn Leu Asp  
 1025 1030 1035 1040  
 Arg Ile Asn Ser Ser Asn Asp Ile Asp Phe Lys Leu Ile Asn Cys Thr  
 1045 1050 1055

Asp Thr Thr Lys Phe Val Trp Ile Lys Asp Phe Asn Ile Phe Gly Arg  
 1060 1065 1070  
 Glu Leu Asn Ala Thr Glu Val Ser Ser Leu Tyr Trp Ile Gln Ser Ser  
 1075 1080 1085  
 Thr Asn Thr Leu Lys Asp Phe Trp Gly Asn Pro Leu Arg Tyr Asp Thr  
 1090 1095 1100  
 Gln Tyr Tyr Leu Phe Asn Gln Gly Met Gln Asn Ile Tyr Ile Lys Tyr  
 1105 1110 1115 1120  
 Phe Ser Lys Ala Ser Met Gly Glu Thr Ala Pro Arg Thr Asn Phe Asn  
 1125 1130 1135  
 Asn Ala Ala Ile Asn Tyr Gln Asn Leu Tyr Leu Gly Leu Arg Phe Ile  
 1140 1145 1150  
 Ile Lys Lys Ala Ser Asn Ser Arg Asn Ile Asn Asn Asp Asn Ile Val  
 1155 1160 1165  
 Arg Glu Gly Asp Tyr Ile Tyr Leu Asn Ile Asp Asn Ile Ser Asp Glu  
 1170 1175 1180  
 Ser Tyr Arg Val Tyr Val Leu Val Asn Ser Lys Glu Ile Gln Thr Gln  
 1185 1190 1195 1200  
 Leu Phe Leu Ala Pro Ile Asn Asp Asp Pro Thr Phe Tyr Asp Val Leu  
 1205 1210 1215  
 Gln Ile Lys Lys Tyr Tyr Glu Lys Thr Thr Tyr Asn Cys Gln Ile Leu  
 1220 1225 1230  
 Cys Glu Lys Asp Thr Lys Thr Phe Gly Leu Phe Gly Ile Gly Lys Phe  
 1235 1240 1245  
 Val Lys Asp Tyr Gly Tyr Val Trp Asp Thr Tyr Asp Asn Tyr Phe Cys  
 1250 1255 1260  
 Ile Ser Gln Trp Tyr Leu Arg Arg Ile Ser Glu Asn Ile Asn Lys Leu  
 1265 1270 1275 1280  
 Arg Leu Gly Cys Asn Trp Gln Phe Ile Pro Val Asp Glu Gly Trp Thr  
 1285 1290 1295

Glu

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1535 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 108..1526

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATAACC ATG GGC CAT	116
Met Gly His	
1	
CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT	164
His His His His His His His His His Ser Ser Gly His Ile Glu Gly	
5 10 15	
CGT CAT ATG GCT AGC ATG GCT GAC ACA ATT TTA ATA CAA GTT TTT AAT	212
Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Gln Val Phe Asn	
20 25 30 35	
AAT TAT ATT AGT AAT ATT AGT AGT AAT GCT ATT TTA AGT TTA AGT TAT	260
Asn Tyr Ile Ser Asn Ile Ser Ser Asn Ala Ile Leu Ser Leu Ser Tyr	
40 45 50	
AGA GGT GGG CGT TTA ATA GAT TCA TCT GGA TAT GGT GCA ACT ATG AAT	308
Arg Gly Gly Arg Leu Ile Asp Ser Ser Gly Tyr Gly Ala Thr Met Asn	
55 60 65	
GTA GGT TCA GAT GTT ATC TTT AAT GAT ATA GGA AAT GGT CAA TTT AAA	356
Val Gly Ser Asp Val Ile Phe Asn Asp Ile Gly Asn Gly Gln Phe Lys	
70 75 80	
TTA AAT AAT TCT GAA AAT AGT AAT ATT ACG GCA CAT CAA AGT AAA TTC	404
Leu Asn Asn Ser Glu Asn Ser Asn Ile Thr Ala His Gln Ser Lys Phe	
85 90 95	
GTT GTA TAT GAT AGT ATG TTT GAT AAT TTT AGC ATT AAC TTT TGG GTA	452
Val Val Tyr Asp Ser Met Phe Asp Asn Phe Ser Ile Asn Phe Trp Val	
100 105 110 115	
AGG ACT CCT AAA TAT AAT AAT AAT GAT ATA CAA ACT TAT CTT CAA AAT	500
Arg Thr Pro Lys Tyr Asn Asn Asn Asp Ile Gln Thr Tyr Leu Gln Asn	
120 125 130	
GAG TAT ACA ATA ATT AGT TGT ATA AAA AAT GAC TCA GGA TGG AAA GTA	548
Glu Tyr Thr Ile Ile Ser Cys Ile Lys Asn Asp Ser Gly Trp Lys Val	
135 140 145	
TCT ATT AAG GGA AAT AGA ATA ATA TGG ACA TTA ATA GAT GTT AAT GCA	596
Ser Ile Lys Gly Asn Arg Ile Ile Trp Thr Leu Ile Asp Val Asn Ala	
150 155 160	
AAA TCT AAA TCA ATA TTT TTC GAA TAT AGT ATA AAA GAT AAT ATA TCA	644
Lys Ser Lys Ser Ile Phe Phe Glu Tyr Ser Ile Lys Asp Asn Ile Ser	
165 170 175	
GAT TAT ATA AAT AAA TGG TTT TCC ATA ACT ATT ACT AAT GAT AGA TTA	692
Asp Tyr Ile Asn Lys Trp Phe Ser Ile Thr Ile Thr Asn Asp Arg Leu	
180 185 190 195	
GGT AAC GCA AAT ATT TAT ATA AAT GGA AGT TTG AAA AAA AGT GAA AAA	740
Gly Asn Ala Asn Ile Tyr Ile Asn Gly Ser Leu Lys Lys Ser Glu Lys	
200 205 210	

ATT TTA AAC TTA GAT AGA ATT AAT TCT AGT AAT GAT ATA GAC TTC AAA Ile Leu Asn Leu Asp Arg Ile Asn Ser Ser Asn Asp Ile Asp Phe Lys 215 220 225	788
TTA ATT AAT TGT ACA GAT ACT ACT AAA TTT GTT TGG ATT AAG GAT TTT Leu Ile Asn Cys Thr Asp Thr Thr Lys Phe Val Trp Ile Lys Asp Phe 230 235 240	836
AAT ATT TTT GGT AGA GAA TTA AAT GCT ACA GAA GTA TCT TCA CTA TAT Asn Ile Phe Gly Arg Glu Leu Asn Ala Thr Glu Val Ser Ser Leu Tyr 245 250 255	884
TGG ATT CAA TCA TCT ACA AAT ACT TTA AAA GAT TTT TGG GGG AAT CCT Trp Ile Gln Ser Ser Thr Asn Thr Leu Lys Asp Phe Trp Gly Asn Pro 260 265 270 275	932
TTA AGA TAC GAT ACA CAA TAC TAT CTG TTT AAT CAA GGT ATG CAA AAT Leu Arg Tyr Asp Thr Gln Tyr Tyr Leu Phe Asn Gln Gly Met Gln Asn 280 285 290	980
ATC TAT ATA AAG TAT TTT AGT AAA GCT TCT ATG GGG GAA ACT GCA CCA Ile Tyr Ile Lys Tyr Phe Ser Lys Ala Ser Met Gly Glu Thr Ala Pro 295 300 305	1028
CGT ACA AAC TTT AAT AAT GCA GCA ATA AAT TAT CAA AAT TTA TAT CTT Arg Thr Asn Phe Asn Asn Ala Ala Ile Asn Tyr Gln Asn Leu Tyr Leu 310 315 320	1076
GGT TTA CGA TTT ATT ATA AAA AAA GCA TCA AAT TCT CGG AAT ATA AAT Gly Leu Arg Phe Ile Ile Lys Lys Ala Ser Asn Ser Arg Asn Ile Asn 325 330 335	1124
AAT GAT AAT ATA GTC AGA GAA GGA GAT TAT ATA TAT CTT AAT ATT GAT Asn Asp Asn Ile Val Arg Glu Gly Asp Tyr Ile Tyr Leu Asn Ile Asp 340 345 350 355	1172
AAT ATT TCT GAT GAA TCT TAC AGA GTA TAT GTT TTG GTG AAT TCT AAA Asn Ile Ser Asp Glu Ser Tyr Arg Val Tyr Val Leu Val Asn Ser Lys 360 365 370	1220
GAA ATT CAA ACT CAA TTA TTT TTA GCA CCC ATA AAT GAT GAT CCT ACG Glu Ile Gln Thr Gln Leu Phe Leu Ala Pro Ile Asn Asp Asp Pro Thr 375 380 385	1268
TTC TAT GAT GTA CTA CAA ATA AAA AAA TAT TAT GAA AAA ACA ACA TAT Phe Tyr Asp Val Leu Gln Ile Lys Lys Tyr Tyr Glu Lys Thr Thr Tyr 390 395 400	1316
AAT TGT CAG ATA CTT TGC GAA AAA GAT ACT AAA ACA TTT GGG CTG TTT Asn Cys Gln Ile Leu Cys Glu Lys Asp Thr Lys Thr Phe Gly Leu Phe 405 410 415	1364
GGA ATT GGT AAA TTT GTT AAA GAT TAT GGA TAT GTT TGG GAT ACC TAT Gly Ile Gly Lys Phe Val Lys Asp Tyr Gly Tyr Val Trp Asp Thr Tyr 420 425 430 435	1412
GAT AAT TAT TTT TGC ATA AGT CAG TGG TAT CTC AGA AGA ATA TCT GAA Asp Asn Tyr Phe Cys Ile Ser Gln Trp Tyr Leu Arg Arg Ile Ser Glu 440 445 450	1460



AAT ATA AAT AAA TTA AGG TTG GGA TGT AAT TGG CAA TTC ATT CCC GTG 1508  
 Asn Ile Asn Lys Leu Arg Leu Gly Cys Asn Trp Gln Phe Ile Pro Val  
                   455                                  460                                  465

GAT GAA GGA TGG ACA GAA TAACTCGAG 1535  
 Asp Glu Gly Trp Thr Glu  
                   470

(2) INFORMATION FOR SEQ ID NO:79:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 473 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

Met Gly His His His His His His His His His His Ser Ser Gly His  
   1                                  5                                  10                                  15  
 Ile Glu Gly Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Gln  
                   20                                  25                                  30  
 Val Phe Asn Asn Tyr Ile Ser Asn Ile Ser Ser Asn Ala Ile Leu Ser  
                   35                                  40                                  45  
 Leu Ser Tyr Arg Gly Gly Arg Leu Ile Asp Ser Ser Gly Tyr Gly Ala  
   50                                  55                                  60  
 Thr Met Asn Val Gly Ser Asp Val Ile Phe Asn Asp Ile Gly Asn Gly  
   65                                  70                                  75                                  80  
 Gln Phe Lys Leu Asn Asn Ser Glu Asn Ser Asn Ile Thr Ala His Gln  
                   85                                  90                                  95  
 Ser Lys Phe Val Val Tyr Asp Ser Met Phe Asp Asn Phe Ser Ile Asn  
                   100                                  105                                  110  
 Phe Trp Val Arg Thr Pro Lys Tyr Asn Asn Asn Asp Ile Gln Thr Tyr  
                   115                                  120                                  125  
 Leu Gln Asn Glu Tyr Thr Ile Ile Ser Cys Ile Lys Asn Asp Ser Gly  
   130                                  135                                  140  
 Trp Lys Val Ser Ile Lys Gly Asn Arg Ile Ile Trp Thr Leu Ile Asp  
   145                                  150                                  155                                  160  
 Val Asn Ala Lys Ser Lys Ser Ile Phe Phe Glu Tyr Ser Ile Lys Asp  
                   165                                  170                                  175  
 Asn Ile Ser Asp Tyr Ile Asn Lys Trp Phe Ser Ile Thr Ile Thr Asn  
                   180                                  185                                  190  
 Asp Arg Leu Gly Asn Ala Asn Ile Tyr Ile Asn Gly Ser Leu Lys Lys  
                   195                                  200                                  205  
 Ser Glu Lys Ile Leu Asn Leu Asp Arg Ile Asn Ser Ser Asn Asp Ile  
   210                                  215                                  220

Asp	Phe	Lys	Leu	Ile	Asn	Cys	Thr	Asp	Thr	Thr	Lys	Phe	Val	Trp	Ile	225	230	235	240
Lys	Asp	Phe	Asn	Ile	Phe	Gly	Arg	Glu	Leu	Asn	Ala	Thr	Glu	Val	Ser	245	250	255	
Ser	Leu	Tyr	Trp	Ile	Gln	Ser	Ser	Thr	Asn	Thr	Leu	Lys	Asp	Phe	Trp	260	265	270	
Gly	Asn	Pro	Leu	Arg	Tyr	Asp	Thr	Gln	Tyr	Tyr	Leu	Phe	Asn	Gln	Gly	275	280	285	
Met	Gln	Asn	Ile	Tyr	Ile	Lys	Tyr	Phe	Ser	Lys	Ala	Ser	Met	Gly	Glu	290	295	300	
Thr	Ala	Pro	Arg	Thr	Asn	Phe	Asn	Asn	Ala	Ala	Ile	Asn	Tyr	Gln	Asn	305	310	315	320
Leu	Tyr	Leu	Gly	Leu	Arg	Phe	Ile	Ile	Lys	Lys	Ala	Ser	Asn	Ser	Arg	325	330	335	
Asn	Ile	Asn	Asn	Asp	Asn	Ile	Val	Arg	Glu	Gly	Asp	Tyr	Ile	Tyr	Leu	340	345	350	
Asn	Ile	Asp	Asn	Ile	Ser	Asp	Glu	Ser	Tyr	Arg	Val	Tyr	Val	Leu	Val	355	360	365	
Asn	Ser	Lys	Glu	Ile	Gln	Thr	Gln	Leu	Phe	Leu	Ala	Pro	Ile	Asn	Asp	370	375	380	
Asp	Pro	Thr	Phe	Tyr	Asp	Val	Leu	Gln	Ile	Lys	Lys	Tyr	Tyr	Glu	Lys	385	390	395	400
Thr	Thr	Tyr	Asn	Cys	Gln	Ile	Leu	Cys	Glu	Lys	Asp	Thr	Lys	Thr	Phe	405	410	415	
Gly	Leu	Phe	Gly	Ile	Gly	Lys	Phe	Val	Lys	Asp	Tyr	Gly	Tyr	Val	Trp	420	425	430	
Asp	Thr	Tyr	Asp	Asn	Tyr	Phe	Cys	Ile	Ser	Gln	Trp	Tyr	Leu	Arg	Arg	435	440	445	
Ile	Ser	Glu	Asn	Ile	Asn	Lys	Leu	Arg	Leu	Gly	Cys	Asn	Trp	Gln	Phe	450	455	460	
Ile	Pro	Val	Asp	Glu	Gly	Trp	Thr	Glu								465	470		

(2) INFORMATION FOR SEQ ID NO:80:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

CGCCATGGCT GACACAATTT TAATACAAGT

30

(2) INFORMATION FOR SEQ ID NO:81:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

GCCTCGAGTT ATTCTGTCCA TCCTTCATCC AC

32

(2) INFORMATION FOR SEQ ID NO:82:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: not relevant

- (ii) MOLECULE TYPE: peptide

- (ix) FEATURE:

- (A) NAME/KEY: Modified-site
  - (B) LOCATION: 12
  - (D) OTHER INFORMATION: /note= "The asparagine residue at this position contains an amide group."

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

Cys	Gln	Thr	Ile	Asp	Gly	Lys	Lys	Tyr	Tyr	Phe	Asn
1				5					10		